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ORIGINAL PAPER

Somatic embryogenesis and shoot organogenesis from leaf explants of *Primulina tabacum*

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Abstracts *Primulina tabacum* is a rare and endangered species that is endemic to China. Establishing an efficient regeneration system is necessary for its conservation and reintroduction. In this study, when leaf explants collected from plants grown in four ecotypes in China are incubated on Murashige and Skoog (MS) medium containing 5.0 μM thidiazuron (TDZ) for 30 days, then transferred to medium containing 5.0 μM 6-benzyladenine (BA), adventitious shoots are then observed. Conversely, when leaf explants are incubated on medium containing 5.0 μM BA for 30 days, then transferred to medium containing 5.0 μM TDZ, somatic embryogenesis is induced. This indicates that somatic embryogenesis and shoot organogenesis could be switched simply by changing the order of two cytokinins supplemented in the culture medium. Histological investigation has revealed that embryogenic cells are induced within 30 days following incubation of explants in medium containing TDZ. Only if embryogenic cells were induced, TDZ could enhance somatic embryogenesis and BA could stimulate shoot organogenesis. When comparing explants from different ecotypes, leaf explants from Zixiadong in Hunan Province could induce low numbers (1–2) of either somatic embryos or adventitious shoots on medium containing either 5.0 μM TDZ or 5.0 μM BA, respectively. Whereas, leaf explants from plants collected from the other three ecological habitats could induce 50–70 somatic embryos/adventitious shoots per explant. Moreover, somatic embryos could induce secondary somatic embryogenesis and adventitious shoots on different media. All regenerated shoots developed adventitious roots when these are transferred to rooting medium, and over 95% of plantlets have survived following acclimatization and transfer to a potting mixture (1:1, sand:vermiculite).

Keywords *Primulina tabacum* Hance · Somatic embryogenesis · Shoot organogenesis · Regeneration

Abbreviations

BA	6-benzyladenine
TDZ	Thidiazuron
NAA	α-naphthalene acetic acid
KIN	Kinetin
IBA	3-indolebutyric acid

Introduction

The species *Primulina tabacum* Hance (Gesneriaceae) was first discovered in the Lianjiang limestone drainage areas in Lianzhou, North Guangdong, China in 1881 (Hance 1883), followed shortly thereafter by its disappearance for more than 120 years. Its recent rediscovery has important scientific value in the study of both ancient and recent climate, soil and co-evolutionary biology of animals and plants in South China in the Five Ridges Region (Ni et al. 2006; Wang et al. 2009; Liang et al. 2010). Now the species

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P. tabacum has been listed as a 'first grade' critically endangered species in China (Peng and Cheng 2002; He and Li 2005). In order to preserve and utilize this rare and endangered plant species, it is absolutely necessary to establish a secure in situ environment and it is prudent to establish an efficient propagation and plant regeneration system in the event of sudden deterioration or loss of the natural environment. We have reported on the micropropagation of *P. tabacum* and also its reintroduction to its natural habitats (Ma et al. 2010; Ren et al. 2010a, b). Those are the first reports on the reintroduction of a rare and endangered plant species via biotechnology in China. Some other scientists have also used biotechnology to conserve and reintroduce rare and endangered plants back into their natural environments (Nikabadi et al. 2010; Bunn et al. 2011). However, micropropagation and reintroduction of micropropagated material could undoubtedly decrease the genetic diversity of plant species which would not benefit its long-term reproduction and conservation. To avoid this, it is necessary to increase the number of plants that are inoculated and extend exploration of its original habitats.

Somatic embryogenesis and shoot organogenesis are different pathways encompassed within in vitro morphogenesis. Different species, genotypes or explants may need different culture media or conditions to induce somatic embryogenesis or shoot organogenesis (Gregory 2004). In *P. tabacum*, somatic embryogenesis and shoot organogenesis have both been successfully induced on different induction media which contained one of the two different cytokinins: thidiazuron (TDZ) and 6-benzyladenine (BA) (Ma et al. 2010). In this report, we increased the number of ecotypes from which explants were derived and investigated some differences related to the induction of somatic embryogenesis and shoot organogenesis from these four habitats. Secondary somatic embryogenesis and adventitious shoot formation were also explored.

Materials and methods

Somatic embryogenesis and shoot organogenesis from leaf explants in vivo

Plants of *P. tabacum* (approximately 10–15 cm high) were harvested in 2007–2008 from four different ecotypes: Dixiahe (Fig. 1a) and Qingjiang (Fig. 1b) in Lianzhou region of the Guangdong Province as well as Xiaguan (Fig. 1c) and Zixiadong (Fig. 1d) in Jiunishan region of the Hunan Province. Among these, plants collected from Dixiahe were the earliest to be discovered in the 1880s by Hance (1883); moreover, plants collected from Zixiadong were the most remote ecotypes from Dixiahe.

Healthy leaves were surface sterilized in 70% (v/v) alcohol for 10 s and 0.1% (w/v) mercuric chloride for 8 min, rinsed with sterile distilled water 3 times, then cut into 1.0 cm^2 explants and placed adaxial surface down on MS basal media (Murashige and Skoog 1962) supplemented with 5.0 μM TDZ for inducing somatic embryogenesis and 5.0 μM BA for inducing shoot organogenesis. All the media contained 30 g l^{-1} sucrose and were adjusted to pH 6.5 and solidified with 0.6% agar (Ma et al. 2010). Culture jars are maintained in a controlled environment growth room at 26 ± 2°C and a 12-h photoperiod with low diffuse light (PPFD <10 μmol m^{-2} s^{-1}). The number of induced somatic embryos or adventitious shoots was counted after culture for 80 days (Table 1).

Proliferation of shoots

Clumps of adventitious shoots with 8–10 shoots each were transferred to MS medium containing 5.0 μM BA and 0.5 μM α-naphthaleneacetic acid (NAA) and cultured under a 12-h photoperiod with PPFD <10 μmol m^{-2} s^{-1}.

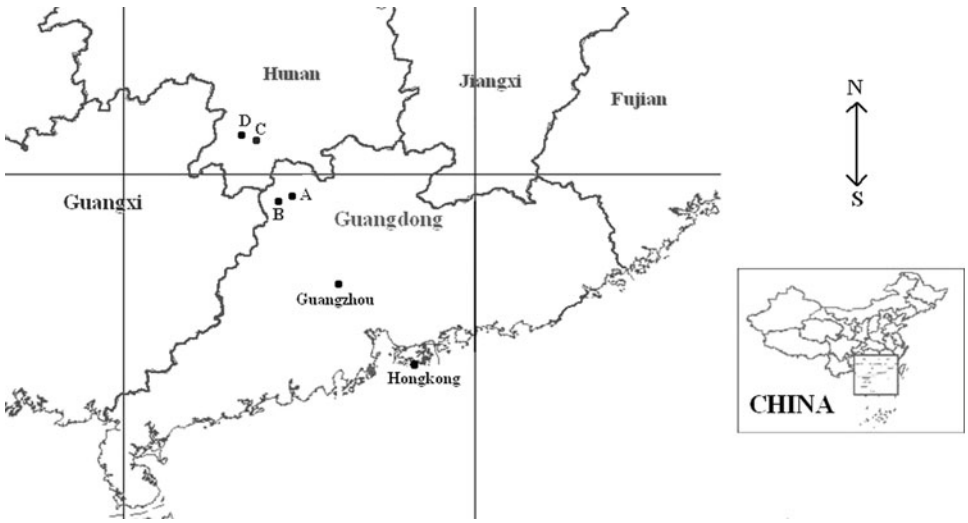

Fig. 1 Four sampled habitats of *P. tabacum*. *A* Dixiahe; *B* Qingjiang; *C* Xiaguan; *D* Zixiadong

Table 1 Somatic embryogenesis and shoot organogenesis in *P. tabacum* from four different habitats

Habitat	Mean number of adventitious shoots/leaf explant on medium containing 5.0 μM BA	Mean number of the somatic embryos/leaf explant on medium containing 5.0 μM TDZ	
		Primary somatic embryo	Secondary somatic embryos
A, Dixiahe	56.8a*	26.3a	21.3a
B, Qingjiang	62.1a	22.1a	18.9a
C, Xiaguan	64.9a	28.2a	22.7a
D, Zixiadong	1.8b	2.3b	1.5b

* The same letter within a column indicates no significant difference using the LSD test ($P = 0.05$)

The adventitious shoots were subcultured on the same medium every 40 days.

Induction of secondary somatic embryogenesis and shoot organogenesis

Primary somatic embryos, and in vitro leaves and petioles from the above culture were isolated and inoculated on different induction media, cultured in the dark for 30 days then incubated under dim light (PPFD <10 μmol m^{-2} s^{-1}). After culturing for a total of 60 days, the number of somatic embryos or shoots per explant was recorded. The experiment was repeated once and data was analyzed using the LSD test ($P = 0.05$).

Effect of cytokinins on induction media and duration of the induction period on somatic embryogenesis and shoot organogenesis

In vitro leaves 0.6 cm^2 in size were used as explants and cultured on induction media in the dark. The induction media contained 5.0 μM BA and 5.0 μM TDZ, for induction of shoot organogenesis and somatic embryogenesis, respectively. After culturing for 15, 30 and 45 days in darkness, the leaf explants (or callus clumps) were transferred to other media containing 5.0 μM TDZ, 5.0 μM BA or free of plant growth regulators for further culture with a 12-h photoperiod at low light intensity (PPFD <10 μmol m^{-2} s^{-1}). After culturing for a total of 60–80 days, the number of induced somatic embryos or adventitious shoots were counted. For each treatment the number of explants was 70–100 and all experiments were repeated twice (Table 2). All data were statistically analyzed by one-way AVOVA with a post hoc test (PLSD) used to separate treatment means ($P \leq 0.05$).

Plantlet root formation and transplanting

Single shoots were isolated from multiple shoot clusters and transferred to rooting medium containing 0.5 μM indole-3-butyric acid (IBA) and 0.2% activated carbon for root formation. After 50 days of incubation in light at PPFD = 50 μmol m^{-2} s^{-1}, plantlets were removed from

Table 2 Transition of somatic embryogenesis and shoot organogenesis on different induction media from leaf explants of in vitro *P. tabacum*

PGR in the first medium	Culture time (days)	PGR in the second medium	Observed results	Number of somatic embryos or adventitious shoots/explant
TDZ	15	BA	Shoot organogenesis	26.3a*
TDZ		0	No obvious cell differentiation	0 b
BA		TDZ	Somatic embryogenesis	21.2a
BA		0	No obvious cell differentiation	0 b
TDZ	30	BA	Both somatic embryogenesis and shoot organogenesis	28.9a
TDZ		0	Somatic embryogenesis	25.3a
BA		TDZ	Both somatic embryogenesis and shoot organogenesis	22.1a
BA		0	Shoot organogenesis	28.7a
TDZ	45	BA	Somatic embryogenesis and shoot organogenesis	25.4a
TDZ		0	Somatic embryogenesis	28.7a
BA		TDZ	Somatic embryogenesis and shoot organogenesis	22.9a
BA		0	Shoot organogenesis	21.8a

* The same letter within a column indicates no significant difference using the LSD test ($P = 0.05$)

the jars and roots were washed to free agar. Rooted shoots were transferred to sand:vermiculite (1:1) and acclimatized in a shaded glasshouse at 25 ± 3°C and 90 ± 5% relative humidity. Survival rate and growth were monitored. Three months later, single plantlets were transferred to plastic pots 12 cm high with a potting mixture of humus and pond sludge (1:1) for continuous culture under the same conditions as for initial stages of acclimatization.

Histological investigation of somatic embryogenesis of in vitro-grown leaf explants

To study the ontogeny and development of embryogenic cell masses, somatic embryos and adventitious shoots, leaf explants incubated on induction medium containing 5.0 μM TDZ and 5.0 μM BA, were subjected to histological analysis. The culture jars were cultured in the dark for 30 days then transferred to low-light condition (<10 μmol $m^{-2} s^{-1}$). After culturing for 0, 10, 20, 30, 45 and 60 days, the leaves (or callus clumps) were fixed in FAA (1:1:18, formaldehyde: glacial acetic acid: 70% alcohol) solution and maintained at room temperature for one week, then transferred to 70% alcohol for storage until required for analysis. The samples were stained with hematoxylin at first, then dehydrated in an increasing alcohol series (35%, 50%, 75%, 85%, 95% and pure alcohol), and embedded in paraffin. Transverse sections 8 μm thick were made with a paraffin-compatible microtome (Fourth Shanghai Medicine Manufacturing Co.) and then laid on filter paper with a brush. The sections were placed in parallel on a slide with 0.1% formalin at 40–45°C to allow the sections to stretch. Slides were then placed in a warm box at 60°C. The sections were dewaxed with xylene three times and finally covered with neutral balsam. Sections were observed under a microscope (Olympus SZX12).

Results

Somatic embryogenesis and shoot organogenesis from leaf explants in vivo

Leaf explants seemed not to respond at first when placed on medium supplemented with 5.0 μM TDZ. As the culture time progressed to 60 days, globular-like protuberances protruded from the leaf surface in some of the cultured explants and generally formed somatic embryos (Fig. 2a). As the culture time progressed, more and more somatic embryos were induced on the leaf surface or on leaf edges. Somatic embryos appeared to be globular when first observed then progressed to more mature stages over time. Finally, shoot buds developed from apex of the somatic embryo, but could not develop into seedlings on the same induction medium (Fig. 2a).

When leaf explants were cultured on medium supplemented with 5.0 μM BA for 60 days, many bud-like protuberances were induced on the leaf surface or leaf edge (Fig. 2b). As the culture incubation period increased more and more adventitious shoot buds were induced and some of these developed multiple shoots. The maximum number of adventitious shoots/explant observed exceeded 100 on one leaf explant.

Among the four habitats, leaf explants in three habitats (Fig. 1a, b, c) could induce averagely 50–70 somatic embryos or adventitious shoots (Table 1; Fig. 2a, b). However, only the leaf explants from habitat Zixiadong (Fig. 1d) of Hunan Province could induce very few somatic embryos or adventitious shoots (1–2). Due to its low index of propagation, we could not establish an efficient propagation system for the latter ecotype on the above media.

Proliferation of shoots

When adventitious shoot clumps were transferred to propagation medium containing 5.0 μM BA and 0.5 μM NAA, they developed into multiple shoot clumps. As the culture period was extended to 2 months, some adventitious roots became visible at the base of shoots (Fig. 2c).

Induction of secondary somatic embryogenesis and shoot organogenesis

Primary somatic embryos, leaves and leaf stalks, all from an in vitro source, could be used as explants and cultured on induction media producing almost the same results as previous experiments using leaf explants, i.e. TDZ-induced secondary somatic embryos from primary somatic embryos (Fig. 2d). Adventitious shoots induced by BA could form from primary somatic embryos, leaves and petioles (Fig. 2e–g). However, the number of somatic embryos or adventitious shoots produced was observed to be less than with primary explants sourced from in vivo tissue (Table 1). This result may be related to the size and thickness of explants because explants produced in vitro were much smaller than primary leaf explants sourced from *in planta* tissues.

Effect of cytokinins on the induction media and the duration of the induction period on somatic embryogenesis and shoot organogenesis

As the leaf explants in vitro were cultured on medium containing 5.0 μM TDZ for 15 days and then transferred to medium containing 5.0 μM BA for further culture, some globular somatic embryos became visible within 60 days. As the culture was transferred to medium containing no plant growth regulators, neither somatic embryos nor

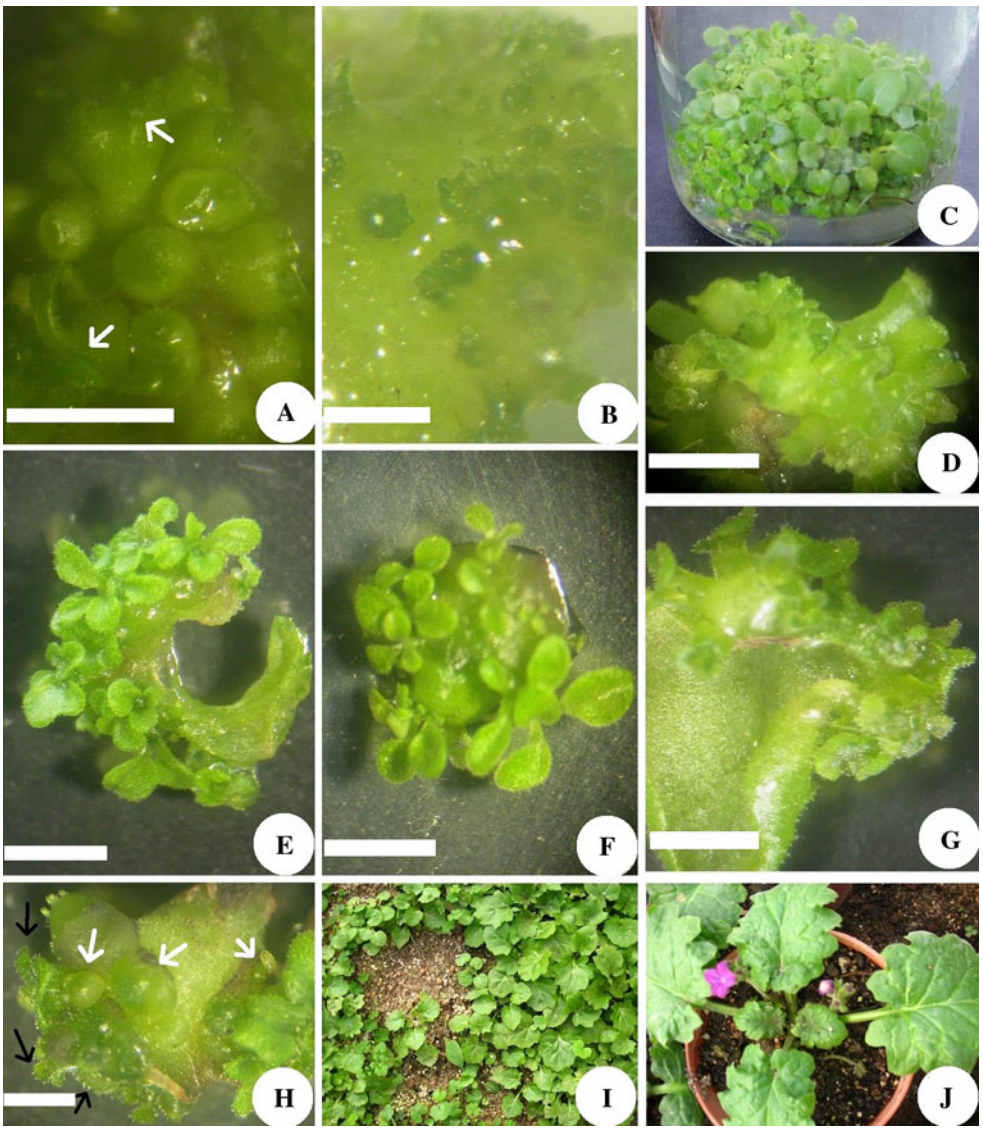

Fig. 2 Somatic embryogenesis and shoot organogenesis in *P. tabacum* (*Bar* = 2 mm). **a** Primary somatic embryogenesis induced from a leaf explant (*white arrows* indicate shoot buds developing at the *top* of somatic embryos) on the induction medium supplemented with 5.0 μM TDZ; **b** adventitious shoots formation induced from one leaf explant on the induction medium supplemented with 5.0 μM BA; **c** multiple shoot propagation on the medium supplemented with 5.0 μM BA and 0.5 μM NAA; **d** secondary somatic embryogenesis induced from a primary somatic embryo on induction medium supplemented with 5.0 μM TDZ; **e** adventitious shoots formation induced from one leaf stalk in vitro on the induction medium supplemented with 5.0 μM BA; **f** Adventitious shoot formation induced from one primary somatic embryo; **g** adventitious shoot formation induced from one leaf explant in vitro on the induction medium supplemented with 5.0 μM BA; **h** Both somatic embryos (white arrows) and adventitious shoots (black arrows)) were induced on the same leaf explant on the induction medium supplemented with 5.0 μM TDZ for 30 days then transferred to the medium containing 5.0 μM BA for continue culture; **i** plantlets in a plastic tray with sand:vermiculite (1:1) potting mixture for 2 months after transplanting from in vitro conditions. **j** flowering of a transplanted plant after 1 year

adventitious shoots could be observed (Table 2). When cultured for 30 days on TDZ-containing medium, some somatic embryos were observed on the leaf explants while after 45 days, both some somatic embryos and adventitious shoots were visible (Table 2).

Similarly, when leaf explants in vitro were cultured on medium containing 5.0 μM BA for 15 days and then transferred to medium containing 5.0 μM TDZ for further culture, some adventitious shoots became visible within 60 days (Fig. 2h). Similarly, as the culture was transferred to medium containing no plant growth regulators, neither somatic embryos nor adventitious shoots were observed on leaf explants (Table 2). When cultured for 30 days on BA-containing medium, some adventitious shoots were

observed on the leaf explants while after 45 days, more adventitious shoots were visible (Table 2).

Plantlet root formation and transplanting

Single shoots were isolated from multiple shoot clusters and transferred to rooting media. Roots formed fastest within 20 days of continuous culture in the light. After incubation for a total of 50 days, rooted shoots were transferred to plastic trays containing sand:vermiculite (1:1). More than 95% of the plantlets could survive and no obvious morphological variation was observed (Fig. 2i). After plantlets were transferred to plastic pots, they could flower normally only after 1 year (Fig. 2j).

Histology of somatic embryogenesis from leaves in vitro

Histological examination showed only 3–4 layers of mesophyll cells in fresh leaves (Fig. 3a). On induction medium with 5.0 μM TDZ or 5.0 μM BA, even when cultured for 10–20 days, there were no embryogenic cell clusters and leaf explants did not become swollen since the layers of mesophyll cells increased (Fig. 3b). After culturing for 30 days, some embryogenic cell masses, darkly stained, were observed (Fig. 3c–e). As culture time was prolonged on induction medium, some globular somatic embryos were observed on the surface of one side of leaf explants when cultured for 40–50 days (Fig. 3f). In a few cases, both sides of leaf explants could induce somatic embryos. As culture time was extended to 50–60 days on induction medium, globular-shaped structures, the somatic embryos, generally developed into heart- or torpedo-shaped somatic embryos (Fig. 3g). The induced somatic embryos could develop into shoot buds when left on the same induction medium (Fig. 3h). After leaf explants were cultured on medium containing 5.0 μM BA for 50–60 days, some adventitious shoots formed (Fig. 3i).

Discussion

For most crops, different subspecies, genotypes or cultivars have obvious and different features and, during morphological induction, they usually show different responses to the same induction medium. In recent years, *P. tabacum* has spread naturally from Guangdong province to neighboring provinces (i.e., Guangxi and Hunan) showing an increasing expansion (Wu et al. 2010). For the wild species, *P. tabacum*, there is no concept of subspecies, genotype or cultivar. In the process of systematic evolution and expansion, they usually varied to adapt to new environments, particularly the plants in Zixiadong (Fig. 1d), which have almost the same leaf morphology, flower color, and ability to grow in a moist limestone drainage environment (and other characteristics) as plants in Dixiahe (Fig. 1a). *P. tabacum* is thus considered as the same taxonomic species (Ni et al. 2006; Liang et al. 2010; Ren et al. 2010a, b). In this tissue culture experiment, *P. tabacum* plants from Zixiadong showed much more differences during induction and propagation than plants from the other three habitats. We thus suggest that *P. tabacum* plants in Zixiadong be classed as different wild ecotypes. This is the first report identifying a new ecotypes via biotechnology and may be a reason why the species has very high levels of genetic differentiation among populations since its populations are very small (Ni et al. 2006; Liang et al. 2010).

In the family Gesneriaceae, somatic embryogenesis or shoot organogenesis have been successfully induced in some species: Leaf explants of *Chirita longgangensis* could directly induce somatic embryos along cut edges on induction medium (MS basal) supplemented with 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA (Tang et al. 2007). In *Chirita medica*, adventitious buds were induced from leaf explants on MS basal medium with 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} BA (Li et al. 2009). In *Aeschynanthus radicans*, leaf explants produced somatic embryos when induction medium (MS) contained 6.81 μM TDZ and 2.68 μM 2,4-D (Cui et al. 2009). In *Sinningia speciosa*, leaf explants were cultured on MS medium containing 2.0 mg l^{-1} BA and 0.2 mg l^{-1} NAA, producing small green calli, after which adventitious buds formed (Xu et al. 2009). In *Metabriggsia ovalifolia*, high activity of cytokinins (TDZ or BA) combined with low activity of auxins (NAA, IAA or IBA) could induce shoot organogenesis from leaves (Ma et al. 2011a). In African violet (*Saintpaulia ionantha*), a high concentration of TDZ (5–10 μM) induce somatic embryogenesis while a low concentration (2.5 μM) induce shoot organogenesis (Mithila et al. 2003; Taha et al. 2009). For *P. tabacum*, 5.0 μM TDZ induced somatic embryogenesis and 5.0 μM BA induced shoot organogenesis (Ma et al. 2010). As expected, all of these studies indicate that different species within the Gesneriaceae need different combinations and concentrations of PGRs for successful organogenesis.

Auxins usually induce somatic embryogenesis. TDZ could also induce somatic embryogenesis in many species indicating that TDZ has a similar effect as auxin with respect to somatic embryogenesis in these species (Ipekci and Gozukirmizi 2003; Zhang et al. 2005; Khan et al. 2006; Kumari et al. 2008). In some cases, TDZ also induce shoot organogenesis (Hosseini-Nasr and Rashid 2002; Feyissa et al. 2005; Ma et al. 2011b, c). In other cases, TDZ induced somatic embryogenesis at high concentrations and induced shoot organogenesis at low concentrations (Dolendro et al. 2003; Mithila et al. 2003; Ma et al. 2011c).

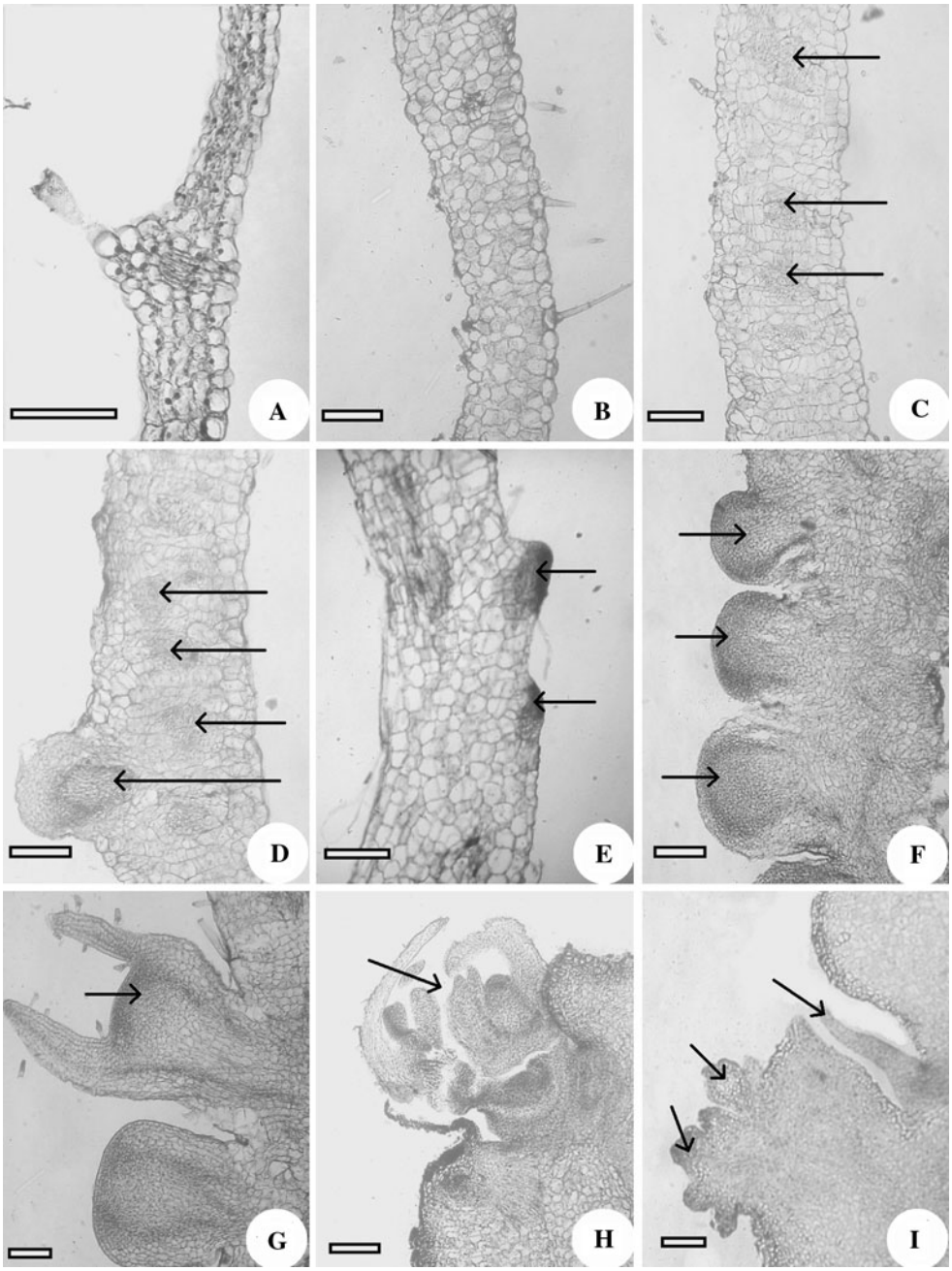

Fig. 3 Light microscopic sections of somatic embryogenesis and shoot organogenesis from leaf explant in vitro in *P. tabacum* (*Bar* = 0.2 mm). **a** Fresh leaf section; **b** leaf explant after culturing for 20 days on the induction medium supplemented with 5.0 μM TDZ or 5.0 μM BA; **c** leaf explant culturing for 30 days showing primordial somatic embryo cell clumps on the induction medium supplemented with 5.0 μM TDZ or 5.0 μM BA; **d**, **e** primordial somatic embryo cell clumps were more visible on the leaf after culturing for 40 days on the induction medium supplemented with 5.0 μM TDZ; **f** globular somatic embryo was visible on the surface of leaf after culturing for 50 days on the induction medium supplemented with 5.0 μM TDZ; **g** heart-stage somatic embryos were observed on both sides of a leaf after culturing for 60 days on the induction medium supplemented with 5.0 μM TDZ; **h** germination of a somatic embryos after culturing for 70 days on the induction medium supplemented with 5.0 μM TDZ; **i** adventitious shoots were visible after culturing for 60–70 days on the induction medium supplemented with 5.0 μM BA

Previous studies indicated that TDZ may act by modulating PGRs, either directly or as a result of induced stress. Other possibilities include the modification of cell membranes, energy levels, nutrient uptake, or nutrient assimilation (Murthy et al. 1998). In all cases, the regeneration pathway maybe either somatic embryogenesis or shoot organogenesis, or both. TDZ is able to alter the pathway of morphogenesis from shoot organogenesis to somatic

embryogenesis through a simple change in TDZ concentration or PGR types (Mithila et al. 2003; Ma et al. 2011c). Our results with *P. tabacum* support the observation that TDZ plays a different role compared with BA. From the results of our histological experiments, during short culture period (less than 30 days), both TDZ and BA could induce were embryogenic cells within 30 days. Only if embryogenic cells were induced, TDZ could enhance somatic embryogenesis and BA could stimulate shoot organogenesis. The embryogenic cells would develop somatic embryos directly as culture time increased. However, once the somatic embryos had been induced, TDZ inhibited their further development into shoots except for a long period culture. During the long culture period (cultured for 30 days or more), once the embryogenic cells were induced, they would develop somatic embryos or adventitious shoots depending on the PGR combination. When leaf explants were cultured on TDZ-contained medium for 30 days, and then transferred to BA-containing media, some somatic embryos and adventitious shoots could both be induced; and in the process of germination and regeneration of somatic embryos, BA also enhanced shoot formation.

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