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Bamboo Regeneration via Embryogenesis and Organogenesis

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1. Introduction

Bamboo is a member of grass family (Poaceae: Bambusoideae) (Wu & Raven, 2006). With the characteristics of short rotation, marketability of culms every year and immediate returns, bamboos are the fast growing multipurpose plants of high economic and environmental value that converts solar radiation into useful goods and services better than most tree species (Franklin, 2006; Kassahun, 2000). Besides producing fresh edible shoots and culms for timber, furniture and handicraft or as a raw material for pulping, bamboo serves as an efficient agent for conservation of water and soil (Christanty et al., 1996, 1997; Kassahun, 2003; Kleinhenz & Midmore, 2001; Mailly et al., 1997). Additionally, new products such as bamboo charcoal, bamboo vinegar, bamboo juice, bamboo healthy food, bamboo fiber product have been developed. World-wide interest in bamboo as a source of biofuel or bioenergy has also increased rapidly in recent years (Fu, 2001; Scurlock, 2000).

There are about 88 genera and 1400 recorded species of bamboo in the world, 34 genera and 534 species of which are in China (Wu & Raven, 2006). Bamboo is found in an area of more than 14 million ha throughout the tropics, subtropics and temperate zones of the world. Eighty percent of the species and area are confined to South and Southeast Asia, and largely in China, India, and Myanmar. China, with the richest resources and largest bamboo industry worldwide, possesses 5 million ha of bamboo forests (Bystriakova et al., 2003; McNeely, 1999).

Because bamboos flowering is unpredictable and has a long interval, the manipulation of bamboo crossbreeding is difficult (Lin et al, 2010a, 2010b; Lu et al., 2009). Gene transformation is another efficient approach to increase productivity and quality in plants. However, there have been no successful reports on bamboo gene transformation till now, because a stable and efficient regeneration system, the prerequisite to bamboo gene transformation, is still not completely established yet (Huang et al., 1989; Zhang et al., 2010; Zuo & Liu, 2004).

2. Process of bamboo regeneration and influencing factor

2.1 Regeneration process

Shoot, seeds, mature zygotic embryo, immature embryo, anther and inflorescence (Figure 1) can be used as explants of bamboo regeneration (Huang & Murashige, 1983; Pei et al.,

2011; Saxena & Dhawan, 1999; Zhang et al., 2010). After inoculation, calli are induced in the medium with a high concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) usually. The quantity and quality of calli differ significantly between different concentration of 2,4-D in the medium. Three kinds of calli are often observed after treated by 2,4-D: (1) Yellowish, granular, and compact calli, with good potential regeneration ability (Fig.2a); (2) Pale-yellow, translucent, watery, and sticky calli, unable to regenerate generally (Fig.2b); (3) Creamy-yellow, compact, and non-embryogenic calli (Fig.2c), unable to regenerate. The yellowish, granular, and compact calli will proliferate after treatment in the callus growth maintenance medium with no or lower levels of 2,4-D compared with callus initiation medium. Then the calli will develop into adventitious shoots, embryo or adventitious roots, after subjected to the differentiated medium, and the callus with adventitious roots (Fig.2d) will not usually continue to differentiate. The destiny of calli is determined by different kinds of plant growth regulators (PGRs) such as 2-isopentenyladenine (2iP), thidiazuron (TDZ), zeatin (ZT), 6-benzyladenine (BA), kinetin (KT), naphthaleneacetic acid (NAA), and indole-3-butyricacid (IBA), etc., and high cytokinin/low auxin will result in adventitious shoot formation. Embryoids have radicles (Fig.2e-g), while adventitious shoot need to root before transplantation. Some shoots will produce roots naturally without any treatment, but others need to be treated with high auxin and minimal or no cytokinin during root development (Fig.2h-i). After rooting, the plantlet can be transferred to potting soil in the greenhouse (Fig.2j). (Huang et al., 1989; Yeh & Chang, 1986a, 1986b, 1987; Zhang et al., 2010).

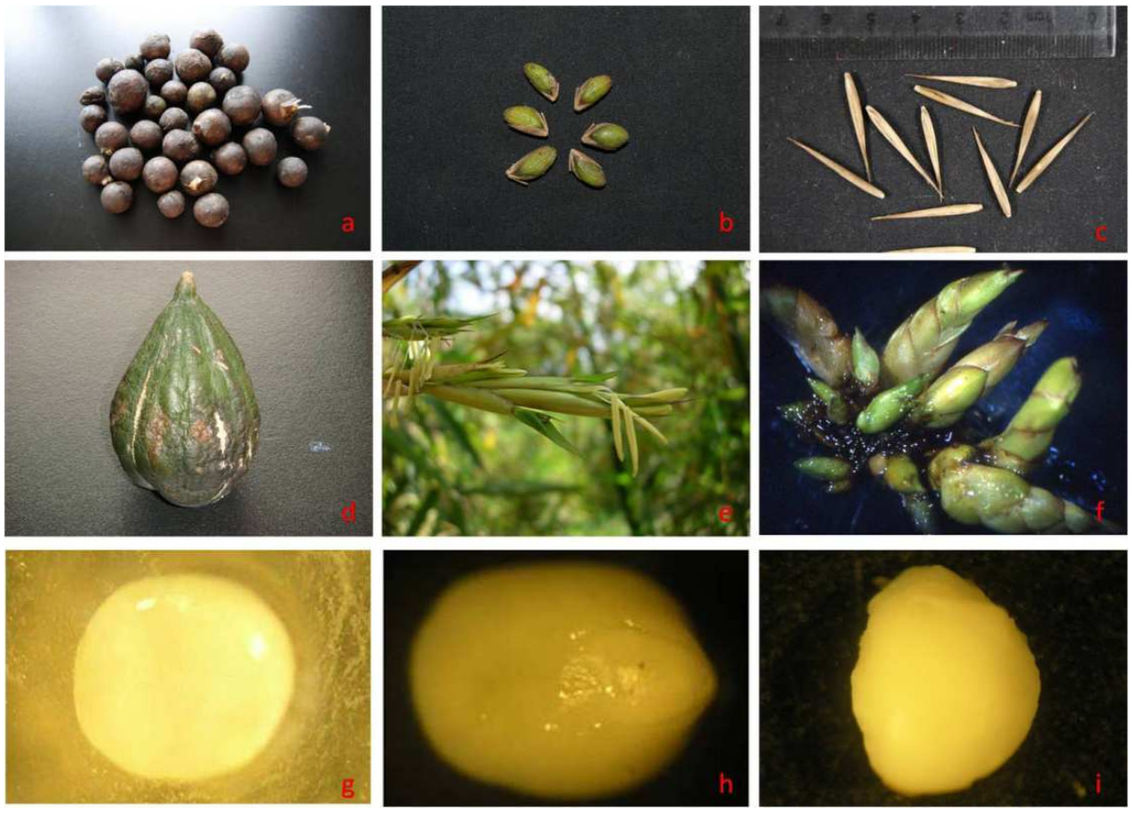

Fig. 1. Explants of bamboos.

a) Seeds of *Melocalamus compactiflorus*; b) Seeds of *Qiongzhurea tumidinoda*; c) Seeds of *Phyllostachys edulis*; d) The seed of *Melocanna baccifera*; e) The inflorescence of *Phyllostachys violascens*; f) Flower buds proliferation of *Bambusa oldhamii*; g) The globular embryo of *Phyllostachys violascens*; h) The cotyledon embryo of *Phyllostachys violascens*; i) The mature embryo of *Dendrocalamus hamiltonii*.

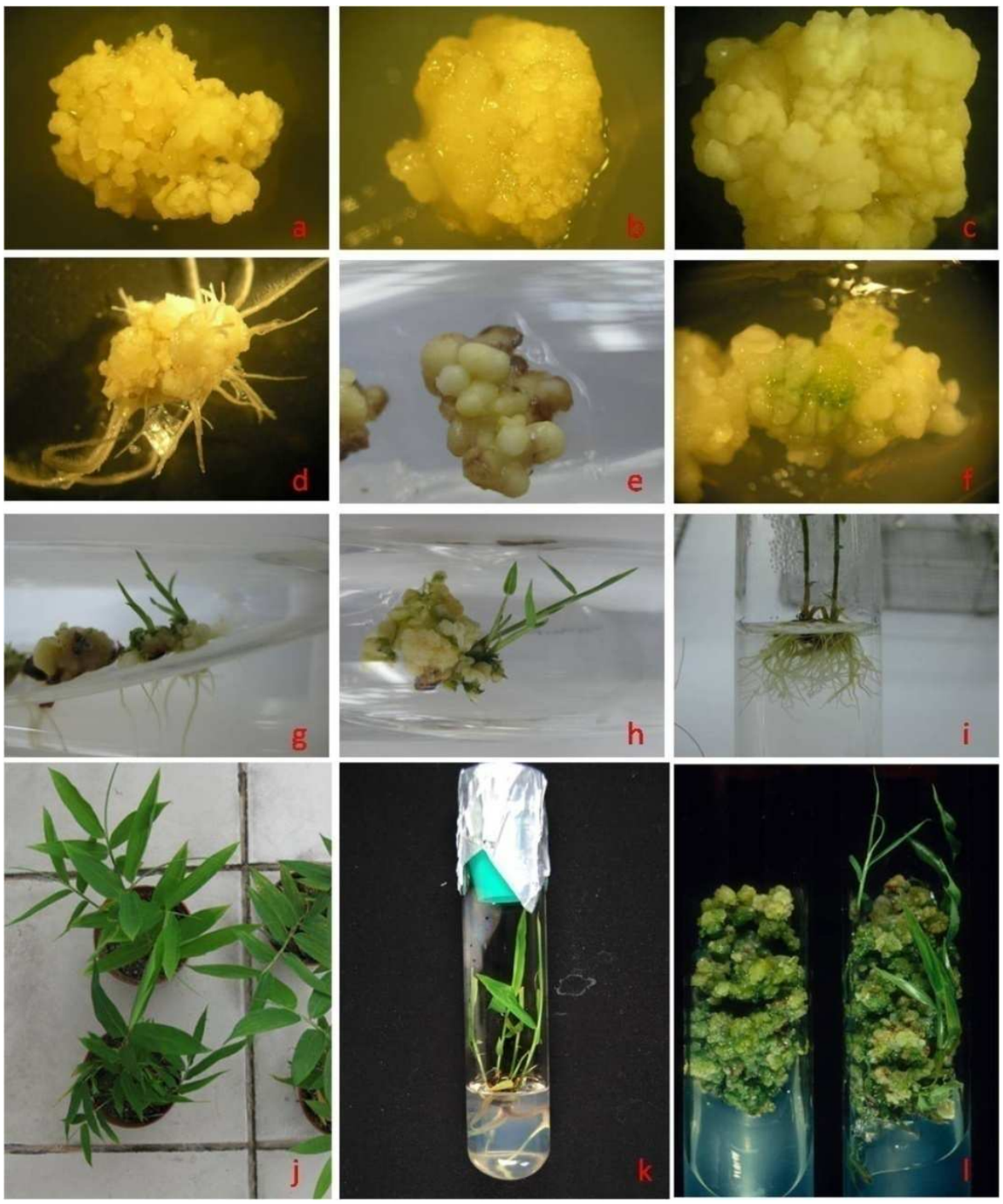

Fig. 2. The somatic embryogenesis and organogenesis of Bamboos.

a-c) Three kinds of calli of *Dendrocalamus hamiltonii*; d) Adventitious roots differentiation of *Dendrocalamus hamiltonii*; e-g) Embryogenesis of *Dendrocalamus hamiltonii*; h-i) Organogenesis of *Dendrocalamus hamiltonii*; j) Transplanted plantlets of *Dendrocalamus hamiltonii*; k) Organogenesis of *Bambusa oldhamii*; l) Organogenesis of *Phyllostachys aurea*.

2.2 Regeneration type

Bamboo can regenerate via embryogenesis and organogenesis (Fig.2e-k), and the frequency of embryogenesis is lower than that of organogenesis (Ramanayake & Wanniarachchi, 2003; Woods et al., 1992; Zhang et al., 2010).

During the bamboo embryogenesis, the embryoids initiate and develop from somatic cells. Compared with organogenesis, somatic embryogenesis is characterized by the formation of a bipolar structure, which will develop into plumule and radicle (Fig.2e-g). Histological analysis reveals that embryogenic cells are small in size, isodiametric with dense cytoplasm, generally locate along the periphery of calli, distribute in clusters, and intersperse with large parenchymal cells. Somatic embryos gradually developed from the granular onsite to heart-shaped, torpedo-shaped, and final cotyledons (Zhang et al., 2010).

In contrasted to the embryogenesis, organogenesis occurs via apparent shoot meristem or leaf primordial (Fig.2h-i). Histological analysis shows that non-embryogenic cells are large, and vacuolated parenchymal cells contain few plastids (Zhang et al., 2010). Callus will differentiate into adventitious shoots, and develop with subsequent formation of adventitious roots.

2.3 Factors affecting regeneration

The bamboo embryogenesis and organogenesis will be affected by many factors such as bamboo species (including cultivars, genotypes and ecotypes), type and age of explants, type of basal media, type and concentration of plant growth regulators, etc. (Godbole et al., 2002; Hassan & Debergh, 1987). Serious browning and difficult differentiation are popular in bamboo regeneration experiments (Huang & Murashige, 1983).

3. Innovative approaches about bamboo regeneration

To establish a stable and efficient regeneration system of bamboo, some efficient measures are proposed as follows:

3.1 Screening of bamboo species

About 20 bamboo species are used for regeneration system establishment in our lab, and we find that the bamboo regeneration ability differs significantly in different kinds of bamboo species, and the sympodial bamboo is easier to regenerate than monopodial bamboo. There are about 1400 kinds of bamboo species in the world, the successful reports about bamboo regeneration mainly focus on the species of *Bambusa* and *Dendrocalamus* (*Sinocalamus* is the anonymus of *Dendrocalamus*), so we can select the bamboo species which are easy to regenerate for overcoming the obstacle of bamboo gene transformation at first.

In addition to the difference among different kinds of species, genotype has distinct influence on the efficiency of plant regeneration via organogenesis or embryogenesis of various plant species such as soybean (*Glycine max*), rapeseed (*Brassica napus*), rice (*Oryza sativa*), etc. (Akasaka-Kennedy et al., 2005; Bailey et al., 1993; Hoque & Mansfield, 2004). Screening of bamboo genotypes or cultivars with strong regeneration ability may be a good choose for setting up an efficient system for bamboo gene transformation.

3.2 Selection of explants

Mature embryos, immature embryos, shoot tips, leaves, young inflorescences, hypocotyls, flower stalks, cotyledons, anthers and nodal segments are the common explants in plant regeneration. Most of those explants (Fig.1) are also efficient during the bamboo regeneration experiments (Lin et al, 2003, 2004; Rout & Das, 1994; Yuan et al., 2009). Within a species, the age of explants is important, loss of competence is correlated with maturation extent of explants, i.e. extent of differentiation, developmentally immature organs (or less differentiated cells) are most likely to contain morphogenetically competent cells. The regeneration ability of mature embryos, immature embryos, shoot tips and flower buds as explants of bamboo regeneration are tested in our lab. We find that the induction and differentiation of shoot tips of young bamboo seedling are easier than those of adult bamboo plants, and embryos, especially for immature embryos, are more efficient than other kinds of explants. Using the immature embryos (Fig.1g-h) as the explants, we have succeeded in setting up a regeneration system of *Phyllostachys violascens*, a species of monopodial bamboo (Pei et al., 2011). However, the materials related to bamboo flowers and seeds such as embryos, inflorescences and anthers are difficult to get for bamboo seldom flowering, shoots which are easier to obtain would be a better choice as the explants.

3.3 Selection of media

The components of media are also important during bamboo callus induction, callus growth maintenance, shoot differentiation, and root development. MS, NB(including N6's macrosalts, B5's microsalts and organic compounds), N6, HB and B5 basal media have been used in bamboo regeneration, and MS basal medium is the most common (Rao et al., 1985; Sun et al., 1999; Tsay et al., 1990; Wu & Chen, 1987; Zhang et al., 2010).

Different kinds of plant growth regulators are needed in different stage of bamboo regeneration, and auxin and cytokinin are the critical components for the morphogenesis in vitro. Besides, ethylene, abscisic acid (ABA) and brassinosteroid (BR) will also have different effects on embryogenesis and organogenesis (Aydin et al., 2006; Torrizo & Zapata, 1986; Vain et al., 1989).

3.4 Complementary approaches for advancing regeneration ability

3.4.1 *NiR* gene

Nitrate assimilation is an important process in rice regeneration. A quantitative trait loci gene encoding the ferrodoxin-nitrite reductase (*NiR*), an enzyme that catalyzes the reduction of nitrite to ammonium leading to the accumulation of toxic nitrite in culture media, was isolated from the high-regeneration rice strain Kasalath. The level of *NiR* expression in the

Koshihikari, a notorious poor rice line for genetic transformation, may result in lower enzymatic activity, and the enzymatic activity is correlated with the regeneration ability. With the introduction of Kasalath *NiR* gene encoding high enzymatic activity, the regeneration ability of low-regeneration rice strain Koshihikari had been improved (Nishimura et al., 2005). The *NiR* gene cloned from the high-regeneration rice strain Konansou has the similar function with that of Kasalath (Ozawa & Kawahigashi, 2006). However, *NiR* gene based improvement method will be suitable for the major Japonica rice varieties but not Indica rice varieties for having high *NiR* activity (Nishimura et al., 2006). In addition, the *NiR* gene can be used as a selection marker for rice gene transformation (Nishimura et al., 2005; Ozawa & Kawahigashi, 2006). The *NiR* gene isolated from the high–regeneration rice line may be useful in promoting the regeneration capacity during bamboo regeneration and gene transformation.

3.4.2 *ipt* gene

Cytokinins play a vital role in the differentiation process of plants. The isopentenyl transferase (*ipt*) gene, isolated from *Agrobacterium tumefaciens*, encodes for isopentenyltransferase which catalyzes the condensation of adenosine - 5′ - monophosphate and isopentenylpyrophosphate to isopentenyladenosine - 5′ - monophosphate (Akiyoshi et al. 1984). Integrating with the *ipt* gene, a cytokinin biosynthetic gene, the transformed cells present higher concentrations of endogenous cytokinins, and lead to higher frequencies of differentiation and transformation than untransformed control cells (Endo et al., 2002; Lopez-Noguera et al., 2009; Smigocki & Owens, 1988). Overexpression of *ipt* gene driven by a strong constitutive promoter favors plant regeneration, and the transformed plants exhibit abnormal morphogenetic variations such as an increased rate of branching, shorter stem internodes and little or no root formation. These morphogenetic changes can be used as selective markers during gene transformation, but these changes, especially for rooting difficultly, also disturb the normal development of transformed plants (Endo et al., 2001; Molinier et al., 2002; Smigocki & Owens, 1988). These defects derived from overexpression of *ipt* gene can be amended by gene deletor technology to delete the exogenous *ipt* gene. (Luo et al., 2007, 2008). It may be a good choose integrating those genes advancing regeneration frequency such as the *ipt* gene with objective genes during bamboo gene transformation.

In addition to *ipt* gene, a number of genes involved in hormone signal transduction have been indentified to influence the regenerative competence of plant cells for somatic embryogenesis and/or adventitious shoot formation (Sakamoto et al., 2006; Srinivasan et al., 2007, 2011).

*KNOX1*genes regulate the shoot apical meristem differentiation, and upregulate cytokinin biosynthesis and decrease gibberellin accumulation in plants, ectopic expression of *KNOX1* genes induces adventitious shoot regeneration in vitro-cultured explants (Sakamoto et al., 2006; Srinivasan et al., 2011).

Heterologous expression of the *BABY BOOM* (*BBM*) AP2/ERF transcription factor enhances the competence of tissues to undergo organogenesis and somatic embryogenesis (Srinivasan et al., 2007).

Identification and application of those genes regulating plant development and regeneration may lead to new approaches for plant regeneration in vitro.

3.4.3 Chemical additives

Many chemical additives including osmoticums, antioxidants, ethylene inhibitors, etc. have the good effects on plant regeneration, and may be used for increasing the efficiency of bamboo regeneration.

Osmotic pressure is correlated with plant development and differentiation, appropriate treatment by the common osmoticums such as sucrose, mannitol and polyethylene glycol (PEG) enhances the embryogenesis and organogenesis in *Solanum melongena* (Mukherjee et al.,1991), *Brassica napus* (Ferrie & Keller, 2007) and white spruce (*Picea glauca*) (Misra et al., 1993).

Tissue browning is the major problem of bamboo regeneration. Oxidized phenolic compounds produced from the damaged explants will suppress enzyme activity, darken the culture medium, and lead to the death of the explants. Treated with antioxidants and absorbents such as cysteine and ascorbic acid, polyvinylpyrrolidone (PVP) and activated carbon, will alleviate the phenolic oxidation and favor the plant regeneration (Abdelwahd et al., 2008; Sanyal et al., 2005; Toth et al., 1994).

Ethylene plays an important role in plant morphogenesis, and has a negative effect on plant regeneration, ethylene inhibitors such as AgNO3 and AVG promote the callus initiation and plant regeneration in cabbage, tobacco, maize and wheat accordingly. The silver ion is reported to be an inhibitor of ethylene action by competitively binding to ethylene receptors which are located predominantly at the intracellular membrane, while AVG inhibits ethylene biosynthesis directly (Vain et al., 1989; Zhang et al., 1998). In addition to silver ion, other heavy metals including Cu and other ethylene inhibitors (Co and Ni), also significantly facilitate the regeneration and somatic embryogenesis (Purnhauser & Gyulai, 1993; Roustan et al. 1989).

3.4.4 Partial desiccation

Partial desiccation has been reported to accelerate plant organogenesis or embryogenesis and results in high regeneration ability significantly in grape (Gray, 1989), wheat (Cheng et al., 2003), *Brassica napus* (Kott & Beversdorf, 1990), rice (Rance et al., 1994; Saharan et al., 2004; Tsukahara & Hirosawa, 1992) and cassava (Mathews et al., 1993). The possible mechanism about its promotion on plant regeneration capacity may be that partial desiccation terminates the developmental mode and "switches" the embryo into a germination mode (Attree et al., 1991). Partial desiccation not only enhances the plant regeneration efficiency, but also benefits the plant organogenesis or embryogenesis and subsequent differentiated stage, and thus reduces the whole time in plant tissue culture (Rance et al., 1994). In addition to the positive effect on wheat organogenesis or embryogenesis, partial desiccation during co-culture greatly enhances the transformation efficiency through inhibiting the growth of Agrobacterium which will suppress the recovery of wheat tissue, and favoring the transfer DNA (T-DNA) delivery (Cheng et al., 2003). Partial desiccation may be also beneficial in bamboo differentiation.

4. Somaclonal variation

Somaclonal variation is the common phenomenon during plant tissue culture. Being high efficient, time-saving and low cost, somaclonal variation has become a useful tool to creating new germplasms with beneficial economic traits of the breeding process in rice, potato, maize, barley and sugar cane, etc. (Karp et al., 1995; Larkin & Scowcroft, 1981). Somaclonal variation, such as mosaic leaf, albino, etiolated shoots, polyploidization and early flowering in vitro etc., also occurs during bamboo embryogenesis and organogenesis (Fig.3), and the frequency of variation will increase after continuous subculture. Most of those bamboo variants can grow normally, but is generally difficult in root formation (Zhang et al., 2010). Compared with poplar and other economic tree species, the process of bamboo breeding is slower for its peculiar flowering characteristics, screening of somaclonal variants with stable and valuable traits may be an alternative choose for bamboo improvement.

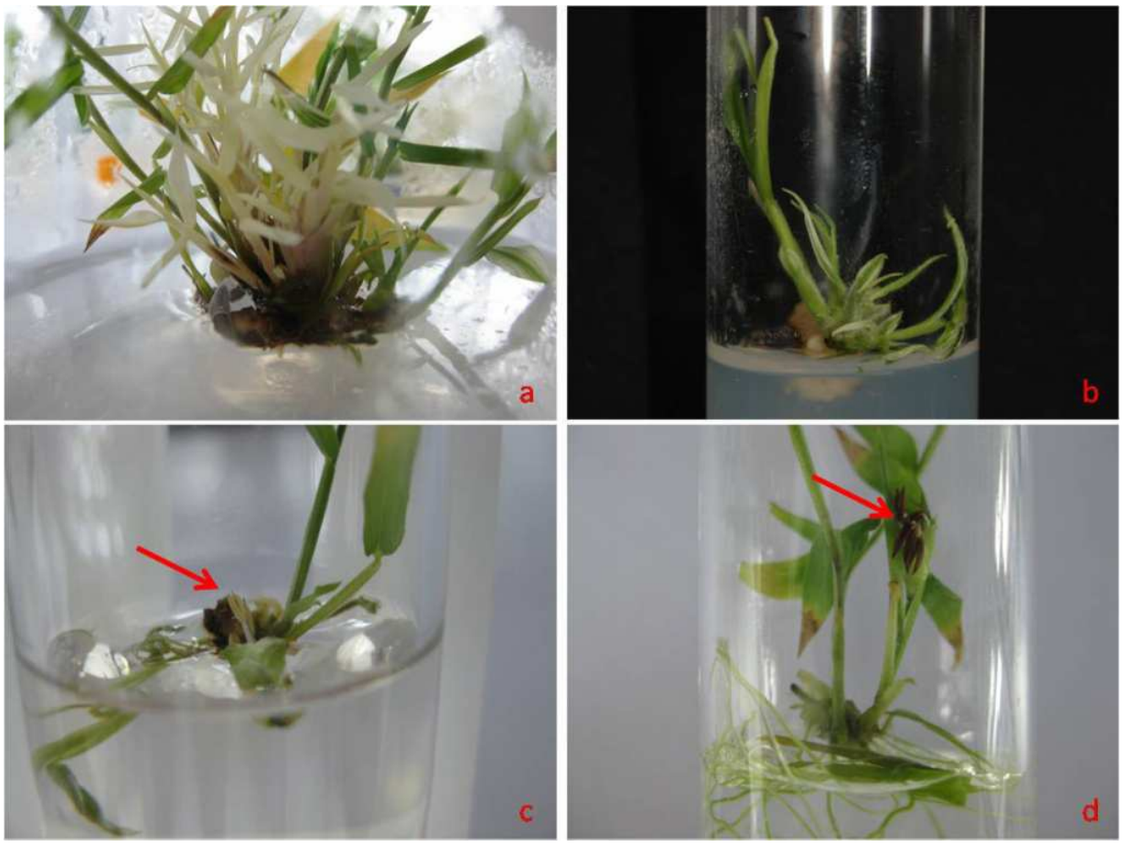

Fig. 3. Somaclonal variation during regeneration of *Dendrocalamus hamiltonii*.

a) Albino plantlets; b) The plantlet with mosaic leaf; c-d) Plantlets flowering in vitro, as indicated with red arrows.

5. Genetic transformation

Using the regeneration system of *Dendrocalamus hamiltonii* of our lab, we tried to establish a bamboo genetic transformation system. After pre-culture for 4 days, good calli were infected

by Agrobacterium strains *EHA105* habouring the *pCAMBIA 1301* vector. After co-culture, for 3 days, the calli were transferred to the recovery medium for 8 days, then the calli were transferred to the selection medium with hygromycin selection. The resistant calli produced and then differentiate shoots and plantlets. To determine the genetic transformation frequency, 10 resistant plantlets were examined by PCR, and all of them are positive, which shows that *Hygromycin B phosphotransferase* (*HPT*) gene of *pCAMBIA 1301* was successfully integrated into the genome of *Dendrocalamus hamiltonii* via agrobacterium, the result was further verified through sequencing the PCR products.

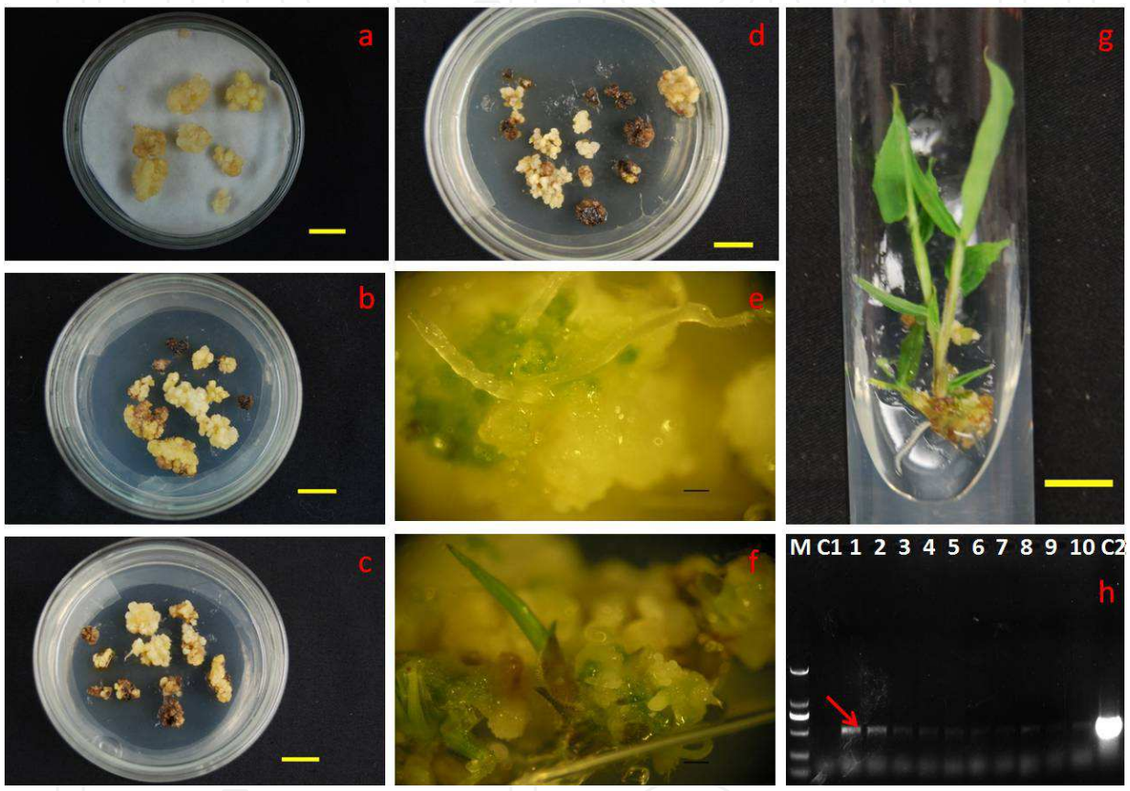

Fig. 4. The genetic transformation and PCR detection of *Dendrocalamus hamiltonii*.

a) Co-culture; b) First selection culture; c) Second selection culture; d) Third selection culture; e-f) Resistant calli differentiate shoots; g) Resistant plantlet; h) PCR detection of plantlets. M: marker; C1: Untransferred plantlet; 1-10: Resistant plantlets; C2: Positive control. Obiective bands were indicated with red arrows.6. Conclusions and prospects

6. Conclusions and prospects

Gene transformation has been proved to be efficient in plant breeding (Nishimura et al., 2006; Varshney et al., 2007). Being one of the most challenging aspects of the gene transformation protocol, a stable and efficient regeneration system must be developed. Lack of a well established regeneration system is the main obstacle for bamboo gene transformation. Understanding bamboo regeneration process and adopting innovative approaches about it will help to enhance the regeneration ability of bamboos and make

breakthrough in bamboo gene transformation. The application of gene transformation will open up a new field for bamboo breeding.

Although we have succeeded in establishing a genetic transformation for *Dendrocalamus hamiltonii* at the first time, there are still many problems during the bamboo genetic transformation process such as serious browning, low differentiate frequency, etc (Zuo & Liu, 2004). More effort is needed to establish a stable and efficient genetic transformation system about bamboos.

7. Acknowledgements

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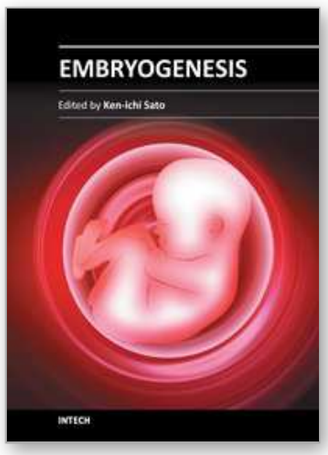

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The book "Embryogenesis" is a compilation of cutting edge views of current trends in modern developmental biology, focusing on gametogenesis, fertilization, early and/or late embryogenesis in animals, plants, and some other small organisms. Each of 27 chapters contributed from the authorships of world-wide 20 countries provides an introduction as well as an in-depth review to classical as well as contemporary problems that challenge to understand how living organisms are born, grow, and reproduce at the levels from molecule and cell to individual.

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