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A method for inflorescence proliferation

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Abstract Most perennial plants must pass through a long juvenile phase of vegetative development before they are capable of flowering. We have developed a method specifying inflorescence proliferation to bypass juvenility and maintain the adult phase. Bamboo (*Bambusa edulis*) inflorescences were amplified by incubation in Murashige and Skoog medium supplemented with 0.1 mg/l thidiazuron. Mutant albino inflorescences also proliferated in this medium. This method is equally effective with dicotyledonous plants. Ginseng (*Panax ginseng*) buds were incubated in B5 medium supplemented with 1 mg/l benzyladenine and 1 mg/l gibberellic acid; new inflorescences developed from the base of the explants. Ginseng flowers were parthenocarpic and some of the fruit proliferated in vitro. Using the inflorescences as the material of somatic embryogenesis, we demonstrated that these were not mutations. The regenerated plants still had a juvenile phase and grew normally.

Keywords Juvenility · Plant tissue culture · Inflorescence proliferation

Abbreviations *BA:* Benzyladenine · *2,4-D:* 2,4-Dichlorophenoxyacetic acid · *GA_3:* Gibberellic acid · *MS:* Murashige and Skoog basal medium · *NAA:* Naphthaleneacetic acid · *TDZ:* Thidiazuron

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Introduction

Flowering is one of the most important processes in plant physiology and agriculture. Control of flowering can improve crop production. One limitation of flowering is juvenility. Many perennial plants must pass through a juvenile phase of vegetative development before they reach the adult phase and are able to flower (Hopkins 1999). Some species, e.g., bamboo, have long juvenile phase. Plant tissue culture can be used as a tool for reducing juvenility (Chang and Hsing 1980; Nadgauda et al. 1990; Lin and Chang 1998). Using a plant tissue culture system, we could micropropagate vegetative shoots, then reduce juvenility and induce flowering. However, in reproductive tissue proliferation, this protocol to proliferate vegetative tissues is expensive in time and money. We wanted to establish a proliferation system that can amplify reproductive organs directly. Such a system would be a useful tool for plant physiological studies and Chinese medicine production.

Bypassing the juvenile phase can have several benefits. It reduces the cost and time normally required to bring plants to the flowering stage, as production of vegetative tissues like shoots and leaves is removed. It also allows researchers to produce flowers year-round.

There are two characteristics of juvenility: (1) branches from the juvenile portion of a shoot express juvenile traits, while adult phase shoots produce adult phase branches (Poeting 1990); (2) auxiliary buds near the roots are more juvenile; axillary buds on the top can maintain adult phase (Janick 1986). In this investigation, we developed a method of in vitro flower proliferation that induces rootless, adult phase, explants to proliferate directly on the medium without passing through a juvenile phase. This method is equally effective with inflorescences of bamboo, a monocot, and adult phase, dormant buds of the dicot ginseng. We use these systems to

investigate the role of medium components in in vitro inflorescence proliferation and growth phase maintenance.

Materials and methods

Plant material

Bamboo explants with multiple shoots were incubated in Murashige and Skoog (1962) (MS) medium supplemented with 0.1 mg/l thidiazuron (TDZ), and subcultured every 21 days (Lin and Chang 1998). Inflorescences were induced on the same medium after 10 months of subculture, and were used for the monocot inflorescence proliferation tests.

Embryoids derived from mature ginseng root callus were cultured in B5 medium (Gamborg et al. 1968) supplemented with 1 mg/l benzyladenine (BA) and 1 mg/l gibberellic acid (GA_3) for germination (Chang and Hsing 1980). The regenerants were grown in the same medium and subcultured every 2 months. The regenerants flowered in this medium and formed dormant buds in the base of explants. Twenty-year-old dormant adult buds were used for the dicot inflorescence proliferation tests.

Direct inflorescences of ginseng were used for inducing somatic embryos. Flowers were cut into 2 mm sections. These sections were plated in MS medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in the dark. Callus was transferred to fresh medium monthly. After 1 year, somatic embryos developed and these embryos were easily separated. These somatic embryos were cultured in B5 medium supplemented with 1 mg/l BA and 1 mg/l GA_3 for germination (Chang and Hsing 1980).

Medium preparation

Bamboo basal medium contained MS salts, 100 mg/l myo-inositol, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine-HCl, 1 mg/l thiamine-HCl, 2 mg/l glycine, 2 g/l Gelrite (Kelco, San Diego, Calif.) and 30 g/l sucrose. The growth regulators were added prior to adjustment of the medium pH to 5.7.

Ginseng basal medium contained B5 salts, 1 mg/l thiamine, 0.1 mg/l pyridoxine-HCl, 0.1 mg/l nicotinic acid, 100 mg/l myo-inositol, 2 g/l Gelrite and 20 g/l sucrose. The growth regulators, except GA_3, were added prior to adjustment of the medium pH to 5.5. GA_3 was filter-sterilized. All media were autoclaved at 121°C for 15 min.

Growth conditions

Bamboo explants were cultured at 26°C with a 16 h photoperiod of 54 μmol m^{-2} s^{-1} artificial light (daylight fluorescent tube, FL-30D/29, 40 W, China Electric, Taipei, Taiwan). Ginseng explants were cultured at 20°C under the same light conditions as bamboo.

Results and discussion

Juvenility and inflorescence proliferation

In previous studies, *Bambusa edulis* exhibited reduced juvenility in vitro on TDZ medium (Lin and Chang 1998). However, this system still required amplification of vegetative shoots, followed by induction of these shoots to flower. In this investigation, bamboo inflorescences were incubated in MS medium supplemented with 0.1 mg/l TDZ and 30 g/l sucrose. New inflorescences developed from the axillary buds of the explants 21 days after subculture (Fig. 1a). These inflorescences developed in TDZ medium; they could also grow in auxin [5 mg/l naphthaleneacetic acid (NAA)] medium. No morphological abnormalities were observed in any in vitro bamboo inflorescences (Fig. 1c). The flowers had anthers with pollen, but the pollen was sterile.

During bamboo micropropagation, an albino mutant was obtained (Lin and Chang 1998). Inflorescences of the albino mutant can also amplify in this medium (Fig. 1b). According to this result, chlorophyll synthesis is not a key factor in adult phase maintenance.

This system can also be applied to dicot plants. We conducted an experiment using 20-year-old dormant adult ginseng buds to determine whether our method of inflorescence proliferation was also effective with dicots. The explants were incubated in B5 medium supplemented with 1 mg/l BA and 1 mg/l GA_3. Some of the buds developed into vegetative shoots (Fig. 2a). In about 15% of buds, new adult inflorescences developed from the base of the bud (Fig. 2b). There were new pistil-like structures amplified from the base of the pistil (Fig. 2c). Fruits formed by ginseng explants were parthenocarpic (Fig. 2d) and some of the fruits proliferated in vitro.

Phase maintenance and inflorescence proliferation

As in the in vivo system, explants (inflorescences) from adult tissues express traits of the adult phase. In thin cell layers of tobacco, explant development is also influenced by developmental phase. Explants from the base produced only (100%) vegetative buds. The buds on explants from floral branches were all (100%) floral (Tran Thanh Van 1973). In the current investigation, bamboo and ginseng adult phase explants (inflorescences) also maintained their adult traits in vitro.

Under physiological conditions, florigen synthesized in response to the photoperiod signal perceived by the leaf is transferred to apical meristems to induce flowering (Hopkins 1999). However, in our system, explants of bamboo and ginseng could flower without leaves in medium containing cytokinins. This indicated that these inflorescences could take up compounds from the medi-

Table 1 Effects of thidiazuron (TDZ) on inflorescence proliferation of *Bambusa edulis*. Data were scored after 21 days, and were means of eight repeats in *1st* subculture experiments. Well growing explants were chosen for *2nd* and *3rd* subculture (four repeats). Each repeat used one florescence

TDZ (mg/l)	Inflorescence proliferation ratio		
	1st subculture	2nd subculture	3rd subculture
0	1.00±0.00[a]	1.00±0.00	1.00±0.00
0.01	1.13±0.35	1.25±0.45	1.00±0.00
0.1	1.63±0.74	2.13±0.22	2.38±0.99
1	1.75±0.89	2.25±1.03	1.84±0.70
10	1.13±0.35	1.25±0.43	1.00±0.00

[a] Mean ±SD

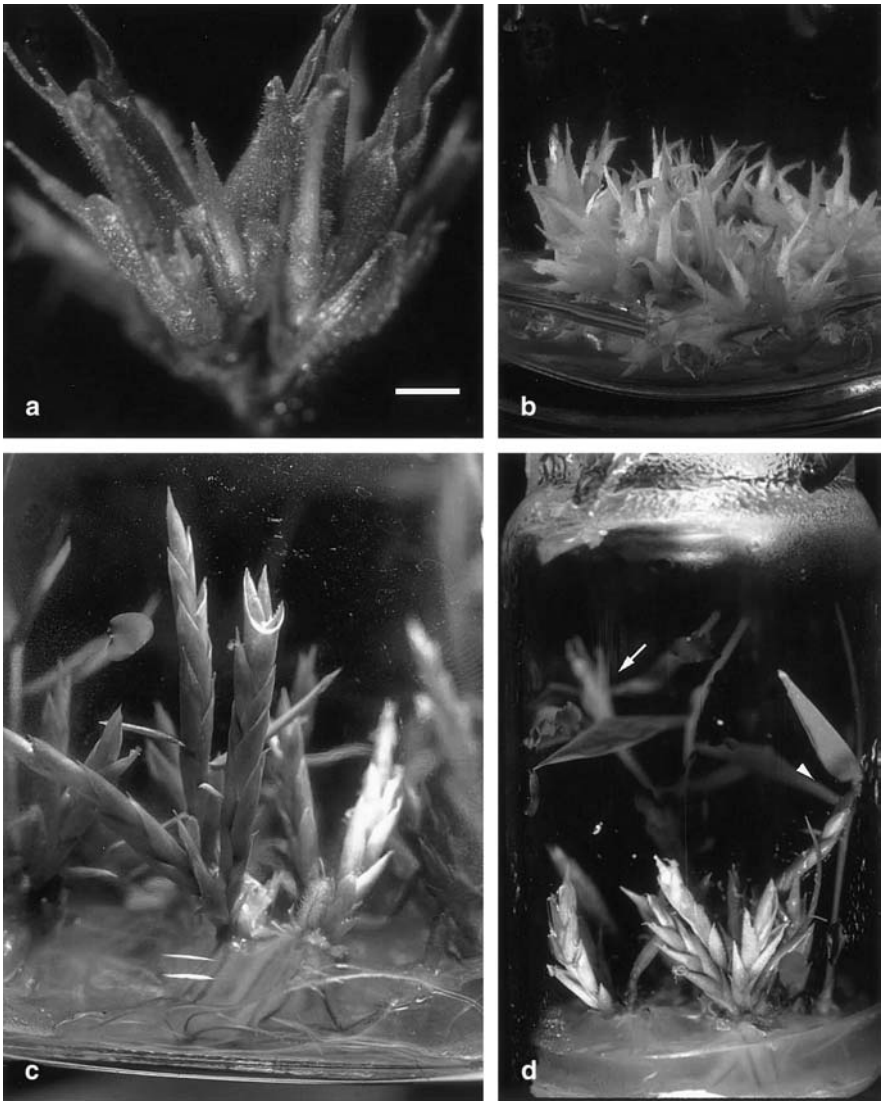

Fig. 1a–d Bamboo inflorescence proliferation. **a** Formation of de novo inflorescences on bamboo explants grown in Murashige and Skoog (MS) medium supplemented with 0.1 mg/l thidiazuron (TDZ). **b** Inflorescence proliferation on albino mutants grown in MS medium supplemented with 0.1 mg/l TDZ. **c** Flower development and adventitious root formation at the base of an inflorescence grown in MS medium supplemented with 5 mg/l naphthaleneacetic acid (NAA). **d** Formation of de novo vegetative shoots (*arrowhead*) by bamboo explants grown in MS medium supplemented with 5 mg/l NAA. One of the shoots is flowering in vitro (*arrow*). *Bars* **a** 2 mm, **b–d** 1 cm

um to synthesize florigen and produce new inflorescences.

TDZ, a urea-derived cytokinin, is a potent cytokinin for woody plant tissue culture (Huetteman and Preece 1993), and is essential for bamboo micropropagation (Lin and Chang 1998). TDZ also maintained the inflorescences in adult phase and induced axillary bud growth. After three rounds of subculture, inflorescences of bamboo could be amplified only in the presence of TDZ, with an optimal concentration of 0.1 mg/l (Table 1). At higher concentration (10 mg/l), inflorescences became brown and did not grow. Treatment with different cytokinins can also induce inflorescence proliferation. In the ginseng system, BA is essential inflorescence proliferation (Lee et al. 1991). These inflorescences can also be amplified in the presence of other cytokinins, like TDZ, in the medium. According to these results, cytokinins are key factors in inflorescence proliferation.

Roots and juvenility

Juvenility is thought to be caused by a substance or substances emanating from the seed or the juvenile root system (Janick 1986). The flower-promoting effect of root pruning in vitro as applied to orchids (Kostenyuk et al. 1999) indicated that roots enhanced juvenility. Our study showed that the growth phase of the explants was ignited by the formation of induced roots. In MS medium supplemented with auxin (5 mg/l NAA), bamboo explants produced adventitious roots but not inflorescences (Fig. 1d). Later, vegetative shoots developed at the base of the explants. We hypothesize that the auxin-induced roots synthesized juvenile factor, transforming the explants from the adult phase to the juvenile phase. As a result, de novo vegetative shoots grew from the base of the inflorescence. However, auxin may induce both root and shoot formation simultaneously. In future, we will design more experiments to test this hypothesis, e.g., root pruning.

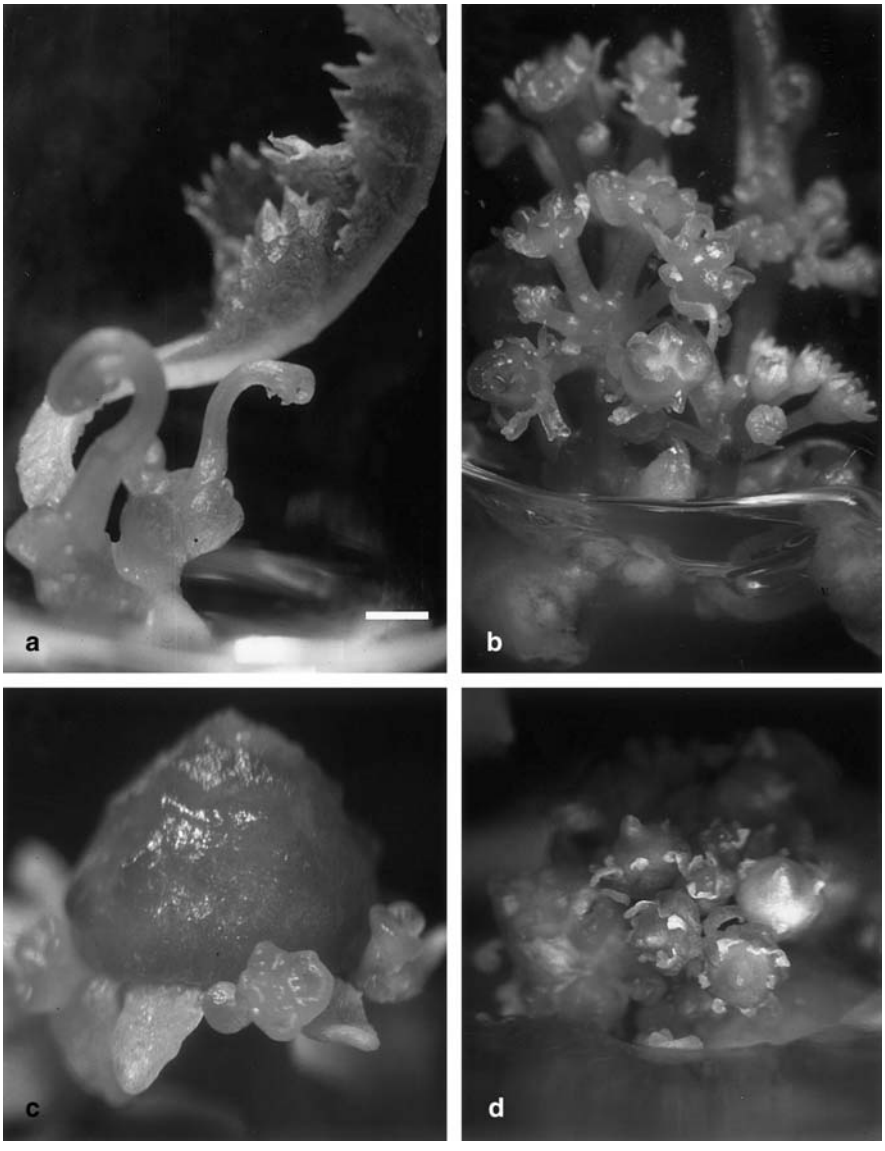

Fig. 2a–d Ginseng inflorescence proliferation. **a** Vegetative shoot formed from a dormant bud grown in B5 medium supplemented with 1 mg/l benzyladenine (BA) and 1 mg/l gibberellic acid (GA_3). **b** An inflorescence with flowers formed directly from an adult phase bud grown in B5 medium supplemented with 1 mg/l BA and 1 mg/l GA_3. **c** New pistils generate from the base of the flower. **d** Parthenocarpic fruit. *Bars* **a**, **b**, **d** 2 mm; **c** 0.5 mm

Effects of gibberellin on inflorescence proliferation

Like cytokinin and auxin, gibberellins also influence juvenility. In some species, the leaf shape and morphology of juvenile plants may differ from that of mature or adult plants. Juvenile branches of English ivy (*Hedera helix*) have long internodes, palmately lobed leaves and do not flower. Adult branches have short internodes, entire, ovate leaves, and do flower. Normally, axillary buds on an adult branch develop into new adult branches. However, treating the bud with GA_3 causes the branch to revert to the non-flowering juvenile form (Hopkins 1999). In our experiments, the effects of gibberellins on inflorescence proliferation were species-dependent. Bamboo inflorescence incubated in MS medium supplemented with GA_3 did not develop normal flowers.

Genes and juvenility

Many genes are involved in the flowering process. Some mutants of these genes can reduce juvenile phase. Loss-of-function mutations in *Arabidopsis EMBRYONIC FLOWER* (*EMF*) genes terminate rosette shoot growth, and transform apical meristem growth from indeterminate to determinate through production of a single terminal flower on the meristem (Sung et al. 1992; Aubert et al. 2001). This mutant phenotype was similar to our explants. They could flower after germination and exhibited no juvenility. In our tissue culture system, we could observe the mutants in subculture. In the bamboo tissue culture system, we isolated an albino mutant (Lin and Chang 1998). We wanted to know whether this inflorescence proliferation system is a physiological phenomenon, or due to a mutation. Using somatic embryogenesis, we determined that the explants used in

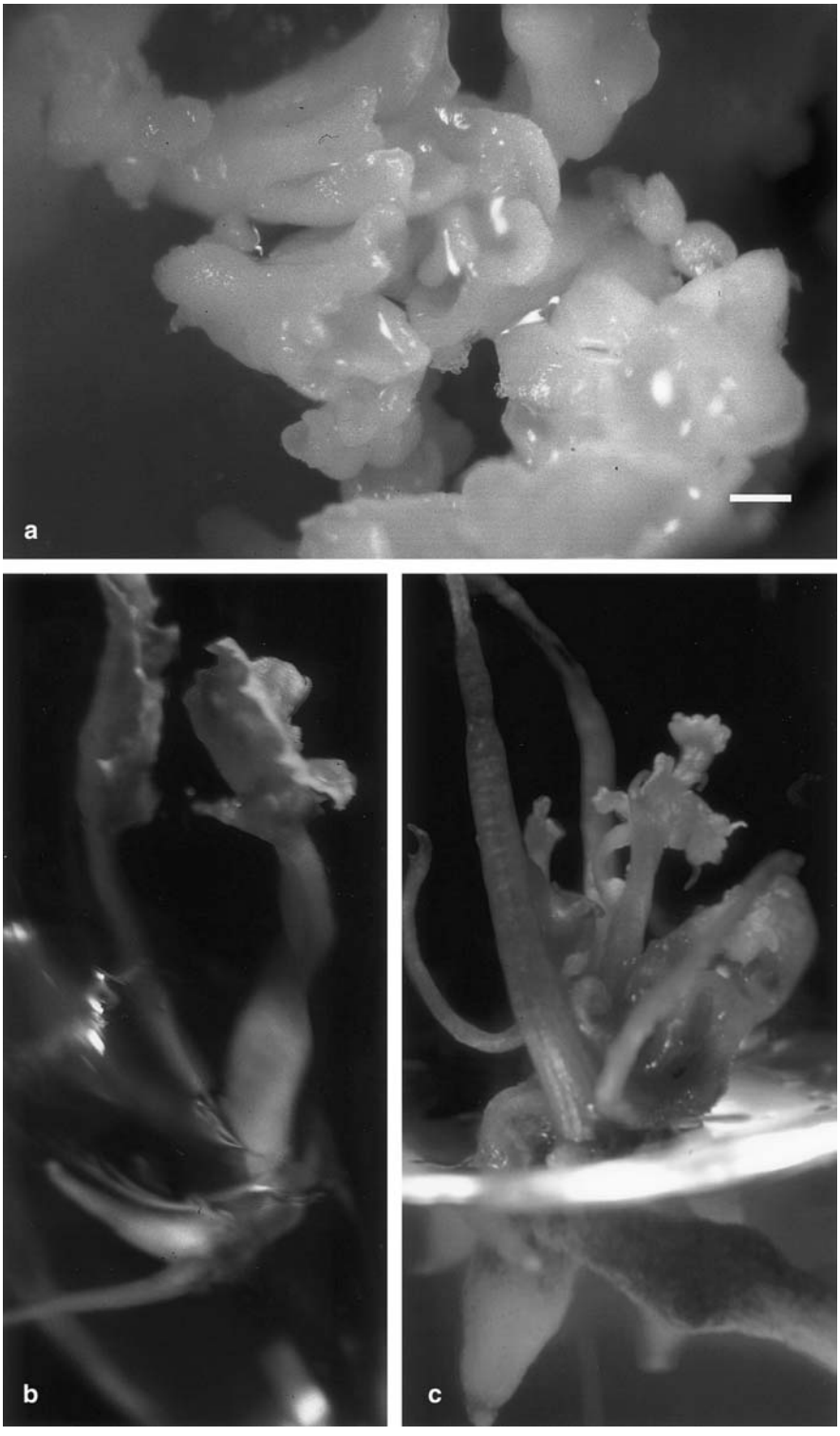

Fig. 3a–c Somatic embryogenesis of ginseng. **a** Direct flowering-bud-derived somatic embryos. **b** Somatic embryo germination in B5 medium supplemented with 1 mg/l BA and 1 mg/l GA_3. **c** Plantlet flowers in B5 medium supplemented with 1 mg/l BA and 1 mg/l GA_3. *Bars* **a** 0.1 mm; **b**, **c** 2 mm

our experiments are not mutations. To induce formation of somatic embryos, in vitro inflorescences were incubated in MS medium supplemented with 2 mg/l kinetin and 3 mg/l 2,4-D for bamboo (Hsu et al. 2000), or MS medium supplemented with 1 mg/l 2,4-D for ginseng (Chang and Hsing 1980), to induce somatic embryos (Fig. 3a). The somatic embryos germinated normally (Fig. 3b), went through a juvenile phase, and developed into complete plants. In a few cases, these plantlets can flower in vitro (Fig. 3c).

Conclusions

Flowering is one of the most important processes in plant physiology and agriculture. Our new plant tissue culture system proved successful in the proliferation of plant reproductive organs and cells. With our method, flowers can be amplified year-round in vitro. Flower propagation under artificial conditions could result in cost and space savings, especially important in medicinal herbs. As the flowers of many Chinese herbs are resources for the medical industry, it is important to reduce the time needed to reach the flowering stage.

In this system, we obtained many bamboo flowers without any vegetative tissue. This material can be used to clone genes specifically expressed in flowers in bamboo. In future, we will use molecular biology tools, e.g., subtraction libraries and microarrays, to isolate such flower-specific genes.

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