Liu, B.L., Yang, Liu, Dong & Wang 2014

Characterization, efficient transformation and regeneration of Chirita pumila (Gesneriaceae), a potential evo-devo model plant.

Plant Cell Tiss Organ Cult doi: 10.1007/s11240-014-0488-2

REFNO: **3888**

KEYWORDS:

Chirita, Cleistogamy, Evo-devo, Genetic transformation, Gesneriaceae

Plant Cell Tiss Organ Cult
DOI 10.1007/s11240-014-0488-2

ORIGINAL PAPER

Characterization, efficient transformation and regeneration of *Chirita pumila* (Gesneriaceae), a potential evo-devo model plant

Bo-Ling Liu · Xia Yang · Jing Liu · Yang Dong · Yin-Zheng Wang

Received: 12 December 2013 / Accepted: 4 April 2014

Abstract An efficient transformation and regeneration system is essential for functional investigation of developmental genes and related elements in the field of evolutionary developmental biology (evo-devo). *Chirita pumila* D. Don belongs to the Gesneriaceae family, one of the most basal groups in Lamiales sensu lato, and possesses many tractable biological features including annual habit, small plant size, short generation time, abundant offspring and low chromosome number. In addition, *C. pumila* has cleistogamous flowers with potential cross-pollination, a special phenomenon first reported herein in Gesneriaceae. Parameters affecting shoot induction and genetic transformation have been evaluated, including plant growth regulators, temperature, antibiotic concentration, pre- and co-culture duration, *Agrobacterium* cell density and infection time. Polymerase chain reaction and β-Glucuronidase (GUS) activity assays of T0 and T1 plants show that the *GUS* gene has been introduced into the host with stable and universal expression. The applicability of the transformation system in gene function investigation is further confirmed by transforming a *GsNST1B* gene from *Glycine soja*. This transformation system provides a valuable platform for deep function analyses of related genes and elements for a wide range of evo-devo studies, especially in the field of floral evolution, which would develop its potential of being a model organism in Lamiales s. l.

Keywords *Chirita pumila* · Cleistogamy · Evo-devo · Genetic transformation · Gesneriaceae

Abbreviations

BA	6-Benzylaminopurine
GFP	Green fluorescent protein
GUS	β-Glucuronidase
*HPT*II	Hygromycin phosphotransferase gene
MS	Murashige and Skoog medium
NAA	1-Naphthaleneacetic acid
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription-PCR

Introduction

Only after the rise of evolutionary developmental biology (evo-devo) has the integration of developmental processes and genetic and evolutionary biology at the molecular level allowed the analysis of how developmental processes can result in morphological evolution (Breuker et al. 2006; Kellogg 2006; Müller 2007; Carroll 2008; Kopp 2009; de Bruijn et al. 2012). Over the past two decades, evo-devo as an emerging biological discipline has made considerable achievements in discovering extensive similarities in gene regulation among distantly related species with dramatically different body plans in both animals and plants relying on rapid technical advancements in gene clone and

Bo-Ling Liu and Xia Yang have contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11240-014-0488-2) contains supplementary material, which is available to authorized users.

B.-L. Liu · X. Yang · J. Liu · Y. Dong · Y.-Z. Wang (✉)
State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, 20 Nanxincun, Xiangshan, Beijing 100093, China
e-mail: wangyz@ibcas.ac.cn

B.-L. Liu
Qufu Normal University, Qufu, Shandong, China

Published online: 20 April 2014

expression. Examples include *Hox* genes in establishing the anterior–posterior axis in bilaterian animals, MADS-box genes in patterning floral organ identities and *CYCLOIDEA* (*CYC*)-like TCP genes in determining floral zygomorphy (Breuker et al. 2006; Kellogg 2006; Müller 2007; Carroll 2008; Kopp 2009; de Bruijn et al. 2012). These studies have provided remarkable insights into the evolutionary conservation of developmental programs and the mechanisms underlying modification of developmental processes that generate morphological novelties (Stern 2000; Pruitt et al. 2003; Breuker et al. 2006). Currently, most evo-devo studies in plants, especially outside model organisms, depend on global DNA sequence analyses and correlative analyses of candidate gene expression to corresponding morphologies rather than gene function investigation. Even though gene expression studies are sufficient ways to screen genes for putative regulatory changes, they are association rather than causality analyses (Baguñà and Garcia-Fernàndez 2003; Kellogg 2004). Therefore, it is essential and critically important to conduct comparative functional studies to demonstrate that such regulatory changes are actually responsible for phenotypic variations and to gain an integrated view of the role of development in evolution (Irish and Benfey 2004; Breuker et al. 2006).

As functional analyses become widespread in evo-devo studies, researchers usually transfer target genes into a distantly related classical model organism to test their function because of the difficulty in carrying out such experiments in native systems (Irish and Benfey 2004). However, these gene transfers are not always efficient to test the genes' function or may not reflect their actual function in native contexts (Irish and Benfey 2004). Therefore, evo-devo biologists have increasingly recognized the limitation of the classical model organisms and the urgency to develop new model organisms to efficiently investigate the genes' actual function in specific morphological novelties (Mandoli and Olmstead 2000; Irish and Benfey 2004; Jeffery 2008).

In angiosperms, one key innovation is the occurrence of the flower with subsequent remarkable diversification upon wide modifications of the genetic programs controlling floral organ identity, floral symmetry and reproduction system (Dilcher 2000; Kramer 2007). Currently, the focus of plant evo-devo studies is mainly on the evolution and diversity of ABCE model beneath floral organ identity and gene network underlying floral symmetry first identified and elaborately studied in classical model species *Arabidopsis* and *Antirrhinum* (Irish and Benfey 2004; Kramer 2007). New evo-devo model organisms would yield new insights into the origin of major floral evolutionary novelties in particular lineage histories that could not be targeted by classical model organisms. The Gesneriaceae family is one of the most basal groups in Lamiales sensu lato (Endress 1998; Cubas 2004; Wortley et al. 2005; http://www.mobot.org/MOBOT/Research/APweb/welcome.html), a major angiosperm clade predominant with zygomorphic flowers that are believed ancestral in this order (Donoghue et al. 1998; Cubas 2004). Therefore, Gesneriaceae locates at a phylogenetic node of floral evolution in angiosperms. As a member of Gesneriaceae, *Chirita pumila* D. Don is a promising candidate of model species for evo-devo studies in floral evolution because it shares a series of biological features with classical model plants, such as annual habit, diploid with the lowest chromosome number ($2n = 8$) in Gesneriaceae (Ratter 1963; Li and Wang 2004; this study), and cleistogamy with potential cross-fertilization (see results in this study). In addition, the whole genome sequencing project is carrying out (Yi-Kun He, personal communication). These unique biological features give *C. pumila* a great advantage in tractability for laboratory experiments over other Gesneriaceae species that are usually perennial and polyploidy with cross-pollination, including the two famous ornamental plants *Saintpaulia ionantha* and *Sinningia speciosa* and the physiological model plant *Ramonda myconi* successful in *Agrobacterium*-mediated genetic transformation (Mercuri et al. 2000; Kushikawa et al. 2001; Tóth et al. 2006; Zhang et al. 2008).

Our laboratory has conducted a series of evo-devo studies relating to the evolution of floral symmetry in Gesneriaceae (Du and Wang 2008; Gao et al. 2008; Zhou et al. 2008; Song et al. 2009; Pang et al. 2010; Yang et al. 2010; Liu et al. 2014) and the molecular mechanism underlying the repeated origins of floral zygomorphy in angiosperms (Yang et al. 2012). Recently, deep functional analyses of related gene networks in floral symmetry and floral organ identity are carrying out in *C. pumila* and its relatives (our unpublished results). Herein, we report an efficient *Agrobacterium*-mediated transformation and regeneration system developed in *C. pumila* by evaluating several factors affecting shoot induction and genetic transformation and validating its applicability in gene function investigation using the *GsNST1B* gene functioning in secondary wall biosynthesis in *Glycine soja*. This transformation system would have wide applications in the field of evo-devo studies.

Materials and methods

Plant material and culture conditions

The *C. pumila* plants, collected from Hekou County, Yunnan, China (Wang, HK01), were grown in 8 cm pots containing the mixture of vermiculite and Pindstrup substrate (Pindstrup) (1:2) in culture room. The growth conditions were: 26 °C, a 10/14 light/dark photoperiod

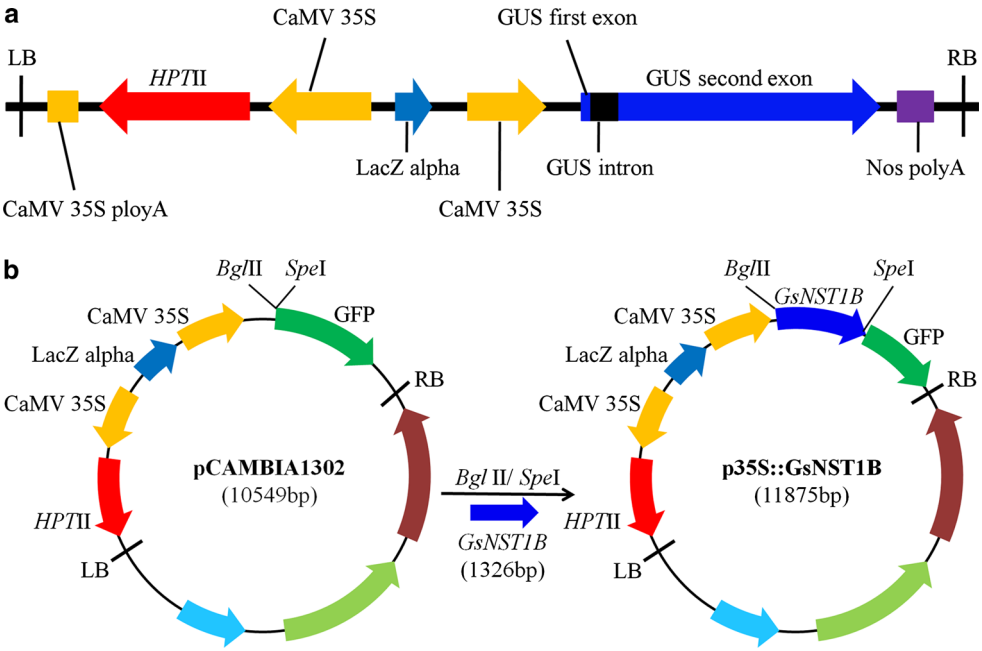

Fig. 1 Map of the binary vector pCAMBIA1301 and the p35S::GsNST1B construct. **a** T-DNA region of the pCAMBIA1301 vector. **b** The binary vector pCAMBIA1302 and the constructed p35S::GsNST1B plasmid

under cool-white fluorescent light of 100 μmol m^{-2} s^{-1} and 50–70 % of relative humidity. Voucher specimens were deposited in the Herbarium, Institute of Botany, Chinese Academy of Sciences.

Agrobacterium strain and plasmids

Agrobacterium tumefaciens strain LBA4404 harboring the binary vector pCAMBIA1301 was used in parameter evaluation experiments and β-Glucuronidase (GUS) activity assay. The vector carries the *hygromycin phosphotransferase* (*HPT*II) gene for transformant selection on hygromycin and the *GUS* reporter gene that is interrupted by an intron (Fig. 1a). The p35S::GsNST1B plasmid was constructed as described (Dong et al. 2013). Briefly, the full-length coding sequence of *GsNST1B* gene (or *GsSHAT1-5*; Dong et al. 2013, 2014) was amplified (5′-GGAAGATCTGCCGGA AAACATGAG-3′ and 5′-GGACTAGTCTACACTG ACG TGTTGGAC-3′), digested with *Bgl*II and *Spe*I, and inserted into the binary vector pCAMBIA1302 that contains the *HPT*II gene and the *green fluorescent protein* (*GFP*) gene (Fig. 1b). The resultant construct was introduced into *Agrobacterium* LBA4404 by electroporation (Eppendorf).

Pollen germination assay and aniline blue staining of pollen tubes

Pollen germination experiment was performed according to Mori et al. (2006). Briefly, pollen was randomly collected from six different flowers close to anthesis and dispersed into sterilized water. 15 μl of the suspension was carefully flattened onto the culture medium (containing 150 g l^{-1} sucrose, 40 mg l^{-1} boric acid, 20 mg l^{-1} calcium chloride, 6 g l^{-1} agar), cultured at 28 °C in the dark for 2–8 h and examined using a Zeiss Axio Imager A1 M Microscope (Zeiss).

To perform aniline blue staining, flowers close to anthesis were either collected directly or bagged in Cellophane for further 2 days. The pistils were dissected and stained with aniline blue according to Jiang et al. (2005). Briefly, the pistils were fixed in ethanol: chloroform: acetic acid (6:3:1) for 24 h, softened in 8 M NaOH for 6 h and washed three times with 0.1 M K_2HPO_4-KOH buffer (pH 7.5). Then, the pistils were stained in 0.1 % aniline blue solution (pH 11) in the dark for 4 h and observed with a Leica TCS SP5 Fluorescence Microscope (Leica).

Karyotype analysis and measurement of the genome size

Root tips were pretreated with a mixture of 2 mM 8-hydroxyquinoline and 0.1 % colchicine (1:1) at 20 °C for 4 h, and fixed in Carnoy's I (100 % ethanol and glacial acetic acid, 3:1) at 5 °C for 1 h. The fixed materials were macerated in 1 M HCl at 60 °C for 1 min, stained with carbol fuchsin, squashed and photographed under a microscope. The length of long and short arms of each metaphase chromosome was measured, and the chromosome arm ratio was estimated by the length of long arm/the length of short arm. The relative length was calculated by the length of individual chromosome/the length of all chromosomes ×100 %.

Flow cytometry was used to measure the genome size of *C. pumila* according to Doležel et al. (2007). Rice (*Oryza sativa* L. var. Nipponbare) was used as an internal standard. Briefly, young leaves of the sample and reference standard were chopped quickly with a sharp razor blade in a plastic Petri dish containing 1 ml ice-cold Galbraith's buffer (45 mM $MgCl_2$, 20 mM MOPS, 30 mM sodium citrate, 0.1 % Triton X-100, pH 7.0) (Galbraith et al. 1983). The resultant homogenate was filtered through a 25 μm nylon mesh to remove large debris, and incubated in staining solution containing 50 g l^{-1} propidium iodide (Sigma-Aldrich) and 50 g l^{-1} RNaseA (TaKaRa) on ice in the dark for 20 min. The relative nuclear DNA fluorescence intensity was measured using a MoFlo® High-performance Cell Sorter (Beckman). Three *C. pumila* plants were analyzed with three replicates each.

Seed germination, shoot induction and antibiotic sensitivity experiments

Chirita pumila plants were grown to flowering stage and seeds were harvested. The seeds were surface-sterilized in 70 % ethanol for 1 min, rinsed with sterile water once, disinfected with 2.5 % sodium hypochlorite for 3–5 min and finally rinsed five times with sterile water. The sterilized seeds were germinated on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) in a growth chamber at 26 °C under a photoperiod of 10/14 h light/dark (100 μmol m^{-2} s^{-1}). About 2-month-old plantlets were used for preparing leaf explants.

To determine optimal concentration of growth regulators for shoot induction, fresh leaf explants were cultured on MS medium containing different concentrations of 6-benzylaminopurine (BA) and α-naphthalene acetic acid (NAA) (see Table 1) at 26 °C. To evaluate the effect of temperature on shoot induction, fresh leaf explants were cultured on MS medium containing 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA (based on the result of growth regulator experiment; see Table 1) at 22, 24, 26 or 28 °C (see Supplementary Table 4). To evaluate whether hygromycin is appropriate for selecting transformants, fresh leaf explants were incubated on MS medium containing 0.5 mg l^{-1} BA, 0.1 mg l^{-1} NAA and different concentrations of hygromycin (0, 5, 10, 15, 20 and 30 mg l^{-1}; see Supplementary Table 5) at 26 °C. For each experiment, the explants were always maintained on the same medium without subculture, and the shoot induction rate was calculated 4 weeks later. Data, presented as mean ± SD, were calculated from three independent experiments with about 40 leaf explants each. Means of induction efficiencies were compared for level of significance ($P < 0.05$) using a Fisher's Least Significant Difference (LSD) test.

Table 1 Effects of different concentrations of BA and NAA on shoot induction rate of *C. pumila* leaf explants

BA (mg l^{-1})	NAA (mg l^{-1})	Shoot induction rate (%)	Root induction rate (%)
0	0	67.8 ± 10.0	0
1	1	72.7 ± 7.3	42.5 ± 11.5
1	0.5	79.9 ± 5.1	15.0 ± 0.6
1	0.2	84.8 ± 8.0	0
1	0.1	87.2 ± 11.8	0
0.5	1	87.6 ± 4.2	92.5 ± 0.3
0.5	0.5	82.2 ± 9.2	42.3 ± 6.7
0.5	0.2	92.5 ± 0.3	0
0.5	**0.1**	**97.6 ± 4.1**	**0**
0.2	1	65.4 ± 13.9	87.4 ± 4.8
0.2	0.5	60.1 ± 8.1	32.2 ± 10.0
0.2	0.2	85.0 ± 0.6	5.0 ± 4.3
0.2	0.1	82.4 ± 4.8	0
0.1	1	89.7 ± 11.8	95.1 ± 4.3
0.1	0.5	59.9 ± 12.2	35.0 ± 3.9
0.1	0.2	89.9 ± 4.6	7.5 ± 0.3
0.1	0.1	74.9 ± 5.0	0

Data are presented as mean ± SD collected from three independent experiments, including a total of 40 leaf explants per replicate

The optimal concentration of BA and NAA for the shoot induction is highlighted by bold letters

Evaluation of parameters affecting shoot induction rate in transformation experiments

Four parameters were successively evaluated, including co-culture time, pre-culture duration, *Agrobacterium* cell density and infection time. In each experiment, only one factor was changed with other fixed. The following is a general protocol for these experiments. Fresh leaf explants were pre-cultured on MS medium containing 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA for 0, 1, 2, 3 or 4 days. *Agrobacterium* LBA4404 cells (harboring pCAMBIA1301) cultured in YEB medium (containing 100 mg l^{-1} streptomycin, 50 mg l^{-1} rifampicin and 50 mg l^{-1} kanamycin) at 28 °C overnight were inoculated to fresh YEB medium and grown to $OD_{600} = 0.2$, 0.4, 0.6, 0.8 or 1.0. The cells were harvested by centrifugation at 5,000 rpm for 10 min, rinsed with MS liquid once, and resuspended in MS liquid containing 150 mg l^{-1} acetosyringone. The harvested cells were used to inoculate pre-cultured explants for 10, 20, 30, 40 or 50 min. After being briefly blot-dried with sterile filter papers, the explants were incubated on the co-culture medium containing 0.5 mg l^{-1} BA, 0.1 mg l^{-1} NAA and 150 mg l^{-1} acetosyringone at 26 °C in the dark for 1, 2, 3, 4 or 5 days, and then transferred to the shoot induction medium containing 0.5 mg l^{-1} BA, 0.1 mg l^{-1} NAA,

20 mg l^{-1} hygromycin and 300 mg l^{-1} carbenicillin. The hygromycin-resistant shoot induction rate was evaluated 4 weeks later. Data (mean ± SD) were calculated from three independent experiments with about 40 leaf explants each. Means of induction efficiencies were compared for level of significance ($P < 0.05$) using a Fisher's LSD test.

GUS activity assay

One-day pre-cultured leaf explants were inoculated with *Agrobacterium* LBA4404 (harboring pCAMBIA1301) for 20 min, cultured on the co-culture medium in the dark for 2 days and then transferred to the shoot induction medium. Hygromycin-resistant shoots of about 0.5 cm in length were excised, transferred to fresh MS medium (without growth regulator and antibiotic), and finally transferred to pots and grown in culture room (the growth conditions were the same as described above). Genomic DNA was isolated from the leaves of putative T0 plants using the Rapid Plant DNA Extraction Kit (Tiangen), and PCR was conducted to identify positive transgenic plants using primers spanning the 35S promoter and the *GUS* gene (5′-GTGAGCGGATAACA ATTTCAC-3′ and 5′-CGAGTCGTC GGTTCTGTAAC-3′). PCR conditions were: 94 °C 3 min, 30 cycles of 94 °C 30 s, 60 °C 30 s and 72 °C 60 s, and 72 °C 10 min. Plasmid and wild-type plants were used as positive and negative controls, respectively. GUS staining was conducted as described (Jefferson et al. 1987). Briefly, leaves and stems of three independent T0 transgenic plants were incubated in GUS staining solution (50 mM sodium phosphate buffer, 0.05 mM potassium ferricyanide, 0.05 % Triton X-100, 2 mM X-Gluc, pH 7.0) at 37 °C overnight and observed under a microscope. Wild-type plants were served as negative controls to exclude the possibility of endogenous GUS expression. To validate whether the transformed *GUS* gene could be inherited, T1 progenies of one T0 plant were disinfected and cultured on MS selection medium containing 25 mg l^{-1} of hygromycin. About 4 weeks later, the segregation ratio was calculated by counting the number of germinated and well developed seedlings and the number of germinated but withered seedlings. The GUS activity assays of T1 progenies were as described above.

Expression and histochemical analyses of *GsNST1B* gene in transgenic plants

Agrobacterium LBA4404 harboring the p35S::GsNST1B plasmid was used to infect *C. pumila* leaf explants. Positive transformants were confirmed by PCR followed by DNA sequencing to exclude the possible amplification of endogenous *NST1B*-like genes. RT-PCR was conducted to measure *GsNST1B* expression in transgenic leaves using specific primers (5′-CTGGCCGCGACAAAGTCATC-3′ and 5′-CTTCTTCCTGAGCAGCATCCG-3′; Dong et al. 2013) under the following conditions: 94 °C 3 min, 30 cycles of 94 °C 30 s, 56 °C 30 s and 72 °C 30 s, and 72 °C 10 min. RT-PCR products were sequenced. As a reference gene, *CpACTIN* was amplified with 26 cycles using specific primers (5′-AGTTATCACCATTGCC GCCGAGAGG-3′ and 5′-GCAATGCCAGGGAACATAGTCGACC-3′). RT-PCR products were visualized on a 1.5 % agarose gel. The ectopic deposition of lignin was examined as described (Dong et al. 2013). Briefly, transgenic leaves were fixed, embedded in Paraplast Plus (Sigma-Aldrich) and stained with 0.2 % toluidine blue solution. The autofluorescence of secondary cell walls was detected using a fluorescence microscope (Zeiss). Three transgenic plants were examined with wild-type ones served as negative controls.

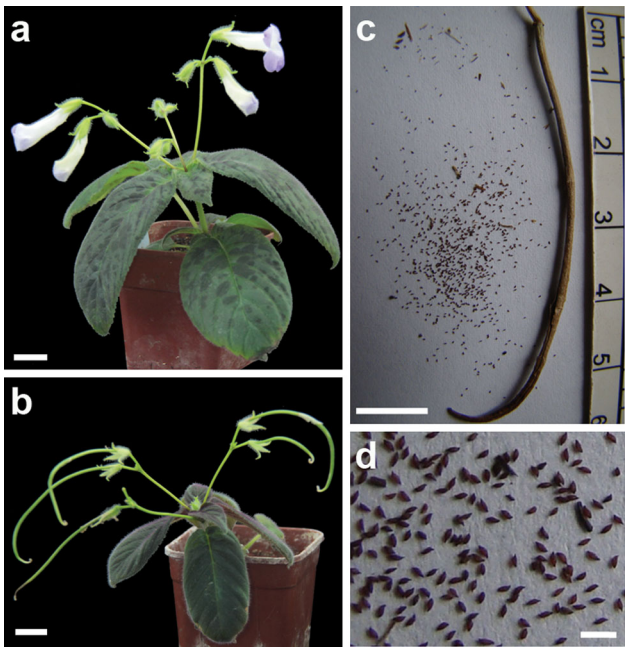

Fig. 2 Morphology of *C. pumila*. **a** A *C. pumila* plant with typical zygomorphic flowers. *Bar*, 2 cm. **b** The capsules of *C. pumila*. *Bar*, 2 cm. **c** The capsule and seeds of *C. pumila*. *Bar*, 1 cm. **d** The enlarged view of **c**. *Bar*, 0.1 cm

Results

Biological characteristics of *C. pumila* plants

Chirita pumila D. Don, an annual herb with erect stems of 6–46 cm in height, extensively distributes in Southwest China, North India, Vietnam, Nepal, Sikkim, Bhutan, Myanmar and Thailand (Wang et al. 1998; Li and Wang 2004). Its typical characteristics include purple-spotted oval leaves and large purplish zygomorphic flowers (Fig. 2a). The capsule is 6–12 cm in length (Fig. 2a, b) that can yield abundant tiny

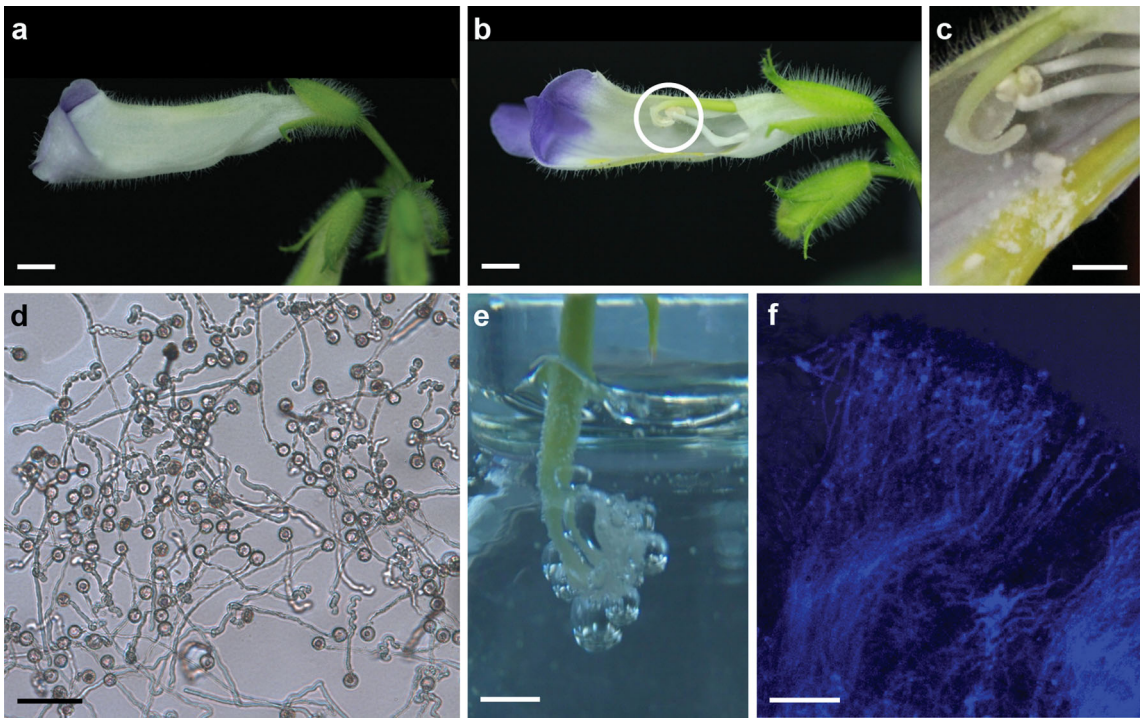

Fig. 3 *C. pumila* flowers are self-pollinated. **a–c** A flower just close to anthesis was dissected longitudinally to show its mature stamens. *Bars*, 0.5 cm. **d** The pollen tubes germinated on culture medium. *Bar*, 100 μm. **e** Activity analysis of the stigma of a flower just before anthesis. *Bar*, 1 cm. **f** Germinated pollen tubes on the stigma of a bagged flower. *Bar*, 200 μm

spindly seeds (over 1,000 seeds per capsule based on a rough estimate) (Fig. 2c, d). In addition, *C. pumila* has a short generation time (about 5 months from seed to seed).

Self-compatibility is critical to a genetic transformation system (Bliss et al. 2013). We have noticed that *C. pumila* flowers always autonomously bear fruits without any source of pollinators in culture room (Fig. 2b). Here, we need to confirm whether and when the *C. pumila* flowers are self-fertile through a series of experiments. We first dissected longitudinally the flowers just close to anthesis to examine whether the sexual organs are mature (Fig. 3a, b). Within the closed corolla tube, the style is held in position pressed against the upper inner surface of the tube with the bilamellar stigma curved downward at the tip (Fig. 3b, c). The filaments of two stamens strongly geniculate at the midpoint and lift the two face-to-face cohered anthers above the stigma and pressing against the style (Fig. 3b, c). There is a great amount of pollen released from anthers and fallen on the lower inner surface of the corolla tube (Fig. 3b, c). The results of in vitro pollen germination experiments further showed that nearly 100 % of pollen grains started to germinate after incubating on the culture medium at 28 °C for 2–3 h, and pollen tubes continued to elongate after 8 h of incubation, indicative of the vitality of pollen (Fig. 3d).

Immersing stigmas in peroxide solution has been used to measure the stigma receptiveness (Bredemeijer 1982). In this experiment, the pistils were dissected from 24 flowers just close to anthesis and immersed into a 3 % H_2O_2 solution for several minutes. Many oxygen bubbles were formed and released from the stigma due to the presence of the peroxidase enzyme (Fig. 3e), indicating the receptivity of the stigma.

To investigate the growth of pollen tubes in situ, flowers close to anthesis were either directly collected or bagged in Cellophane for further 2 days. The pistils were dissected and stained with aniline blue. Under the fluorescent microscope, a great number of pollen grains were found to adhere to the stigmas of the flowers just close to anthesis and the pollen tubes began to germinate (data not shown). The pollen tubes were readily visualized on the stigmas of the bagged flowers (Fig. 3f).

We further tested the self-fertilization of *C. pumila* flowers by bagging experiment. The seed setting percentage was counted 2 weeks later. Of 60 flowers analyzed, 58 yielded fertile capsules (96.7 % of seed setting percentage; see Supplementary Table 1). To examine whether *C. pumila* could be cross-pollinated when flowers open, we artificially emasculated seven immature flowers of about 1.5 cm in length (mature flowers are 3–4 cm in length), and then artificially pollinated six flowers 48–72 h later with one flower served as a negative control. About 2 weeks later, six hand-pollinated flowers all produced

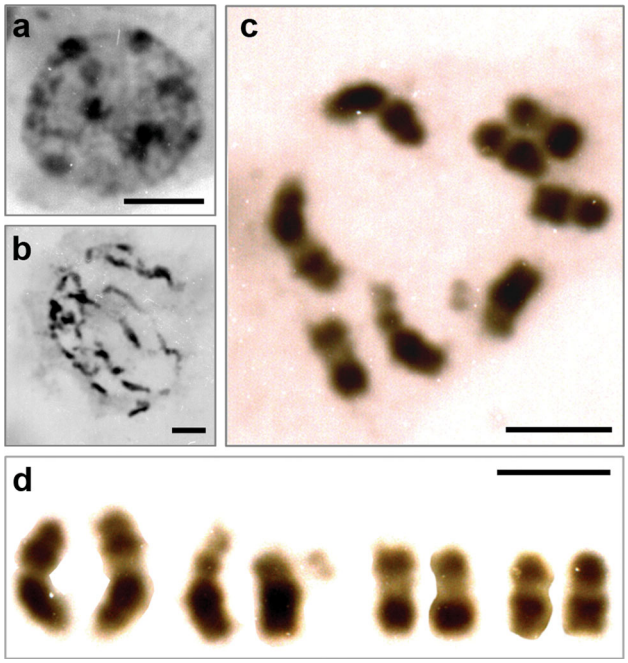

Fig. 4 The karyotype analyses of *C. pumila*. **a** Resting nucleus. *Bar*, 5 μm. **b** Mitotic prophase chromosomes. *Bar*, 5 μm. **c** The eight chromosomes at the mitosis metaphase. Bar, 5 μm. **d** The karyotype of *C. pumila*. *Bar*, 5 μm

fertile capsules, whereas the artificially emasculated but non-pollinated flower failed to fruit (Supplementary Fig. 1), indicating that *C. pumila* flowers have a potential of cross-pollination, facilitating the genetic cross.

Karyotype and genome size analyses of *C. pumila*

The chromosome number and size of *C. pumila* were evaluated by observing root tip cells at the mitotic meta phase under a microscope (Fig. 4a, b). The result showed that *C. pumila* has eight chromosomes of 3.5–6.0 μm in length (Fig. 4c, d). The karyotype is formulated as $2n = 8 = 6\ m + 2\ sm$ (2 sat) with three pairs of m-chromosomes and one pair of sm-chromosomes (Fig. 4d; Supplementary Table 2). The first pair of chromosomes has a secondary constriction in the interstitial region of both the long and short arms (Fig. 4d, No. 1 and 2), whereas the second one has a secondary constriction only in the long arm (Fig. 4d, No. 3 and 4). Two sm-chromosomes have a satellite in the terminal region of the short arm (Fig. 4d, No. 3 and 4).

We further measured the absolute nuclear DNA amount (genome size) of *C. pumila* using flow cytometry. Three different *C. pumila* plants were analyzed with rice (*Oryza sativa* L. var. Nipponbare) served as an internal reference standard. According to Burr (2002), the 2C DNA amount of rice is 0.9 pg. In this experiment, the mean ratio of G1 peaks (*C. pumila* : rice) was 1.8 (Supplementary Fig. 2). Therefore, the 2C DNA amount of *C. pumila* was estimated to be 1.6 pg. According to the formula 1 pg DNA = 0.978×10^9 bp (Doležel et al. 2007), the haploid genome size of *C. pumila* was about 798.7 Mb (Supplementary Table 3).

Shoot induction and antibiotic sensitivity experiments

BA and NAA at different concentrations were added into MS medium to assess their effects on shoot induction rate. Leaf explants thickened after about 1 week of culture. About 2 weeks later, adventitious shoots began to appear on the wound edges of the explants. While MS medium lacking growth regulators led to a relatively low shoot induction rate (67.8 ± 10.0 %), the addition of appropriate concentration of BA and NAA enhanced it. The highest shoot induction rate (97.6 ± 4.1 %) was obtained when 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA were applied (Table 1). Under this condition, abundant adventitious shoots could be induced from explants within 4 weeks with no appearance of undesirable roots (Table 1; Supplementary Fig. 3). The result also showed that relatively lower BA and higher NAA led to high root induction rate, and it reached up to 95.1 ± 4.3 % when 1.0 mg l^{-1} NAA were applied (Table 1; Supplementary Fig. 3). Nevertheless, root induction in *C. pumila* requires neither BA nor NAA, and the adventitious shoots could naturally generate roots after transferring to fresh MS medium without any growth regulator (data not shown). Therefore, 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA were applied in following shoot induction experiments, and MS medium without any growth regulator was used for root induction.

It is reported that abundant adventitious shoots could be induced from two Gesneriaceae plants at 25 °C (Tang et al. 2007a, b). Here, to evaluate whether different temperatures affect the shoot induction rate, fresh leaf explants were cultured on MS medium supplied with 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA at different temperatures. The highest shoot induction rate (97.8 ± 11.6 %) was obtained at 26 °C. Both higher and lower temperatures reduced the shoot induction rate, and it dropped to 55.5 % at 22 °C (Supplementary Table 4). However, Fisher's LSD test ($P < 0.05$) showed that the shoot induction rates obtained at 24, 26 and 28 °C were not significantly different from each other, indicating that *C. pumila* can adapt to a relatively wide temperature range. Nevertheless, for uniformity, an intermediate temperature, i.e. 26 °C was applied in following experiments.

Hygromycin at different concentrations was added into MS medium containing 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA to determine whether it is effective for selecting *C. pumila* transformants. The induction of adventitious shoots was severely affected by hygromycin at the concentration of 10 mg l^{-1} or higher. When its concentration reached to 20 mg l^{-1}, all explants became necrotic with no shoot

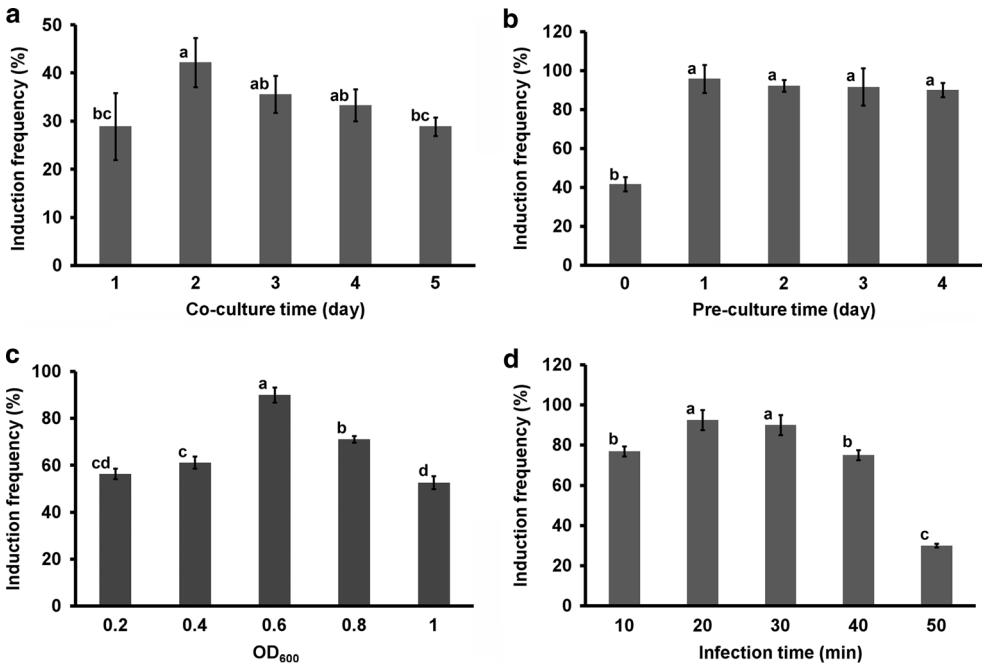

Fig. 5 Effects of different factors on the shoot induction rate of *C. pumila*. Effects of co-culture time **(a)**, pre-culture time **(b)**, OD_{600} value **(c),** and infection time **(d)** on the shoot induction rate were determined. Means of induction efficiencies were compared using a Fisher's LSD test ($P < 0.05$) and column bars labeled with the *same letters* are not significantly different. Data, presented as mean ± SD, were calculated from three independent experiments with about 40 leaf explants each

induced (Supplementary Table 5). Therefore, 20 mg l^{-1} hygromycin was used in following transformation experiments.

Factors affecting hygromycin-resistant shoot induction frequency in transformation experiments

Several factors affect *Agrobacterium* inoculation and shoot induction, including *Agrobacterium* cell density, infection time, pre-culture time and co-culture duration (Mondal et al. 2001; Kim et al. 2004; Barik et al. 2005; Crane et al. 2006; Du and Pijut 2009; Jian et al. 2009). In this study, co-culture time was first examined by infecting fresh leaf explants with *Agrobacterium* LBA4404 of $OD_{600} = 0.6$ for 20 min and culturing on the co-culture medium for 1–5 days. The highest shoot induction rate (42.2 ± 5.1 %) was achieved after 2-days of co-culture, and it declined with shortened or prolonged co-culture (Fig. 5a). 1- and 5-days of co-culture resulted in the lowest shoot induction rate. Hence, co-culture for 2 days was applied to next transformation experiments.

To evaluate whether pre-culture could enhance the induction frequency, newly prepared leaf explants were pre-cultured on MS medium for 0–4 days, inoculated with *Agrobacterium* of $OD_{600} = 0.6$ for 20 min, and co-cultured for 2 days. While fresh explants gave rise to the lowest shoot induction rate (41.7 ± 3.6 %), pre-culture significantly enhanced it (Fig. 5b). 1-day of pre-culture witnessed the highest shoot induction rate (95.8 ± 7.2 %), whereas extended duration led to slightly lower rate. Even though Fisher's LSD test ($P < 0.05$) showed that 1, 2, 3 or 4 days of pre-culture resulted in no significant difference in the shoot induction rate, a pre-culture of 1 day was applied in following assays due to timesaving and high shoot induction rate.

To analyze whether the OD_{600} value of *Agrobacterium* influences the induction frequency, 1-day pre-cultured leaf explants were inoculated with *Agrobacterium* LBA4404 of different OD_{600} values for 20 min and co-cultured for 2 days. The highest regeneration frequency (90.0 ± 3.2 %) was achieved at the late-log phase (corresponding to $OD_{600} = 0.6$), whereas both lower and higher OD_{600} values significantly reduced it (Fig. 5c). When OD_{600} reached up to 1.0, the lowest regeneration efficiency (52.6 ± 2.8 %) was obtained because of the uncontrollable overgrowth of *Agrobacterium*.

To verify if *Agrobacterium* inoculation time affects the shoot induction rate, 1-day pre-cultured leaf explants were immersed in *Agrobacterium* LBA4404 solutions ($OD_{600} = 0.6$) for 10–50 min. 20 min of inoculation was found to achieve the highest shoot induction frequency (92.5 ± 5.0 %) (Fig. 5d). It markedly decreased with increased inoculation time, dropping to 30.0 ± 1.0 % when the infection time was 50 min because of the overgrowth of *Agrobacterium*. Although the difference between 20 and 30 min of inoculation was not significant, a relatively short infection period is probably more beneficial for the viability of explants. Therefore, 20 min of inoculation was used in following experiments.

GUS activity assays

57 Hyg-resistant plantlets from different leaf explants were obtained using the optimal conditions, i.e. inoculating

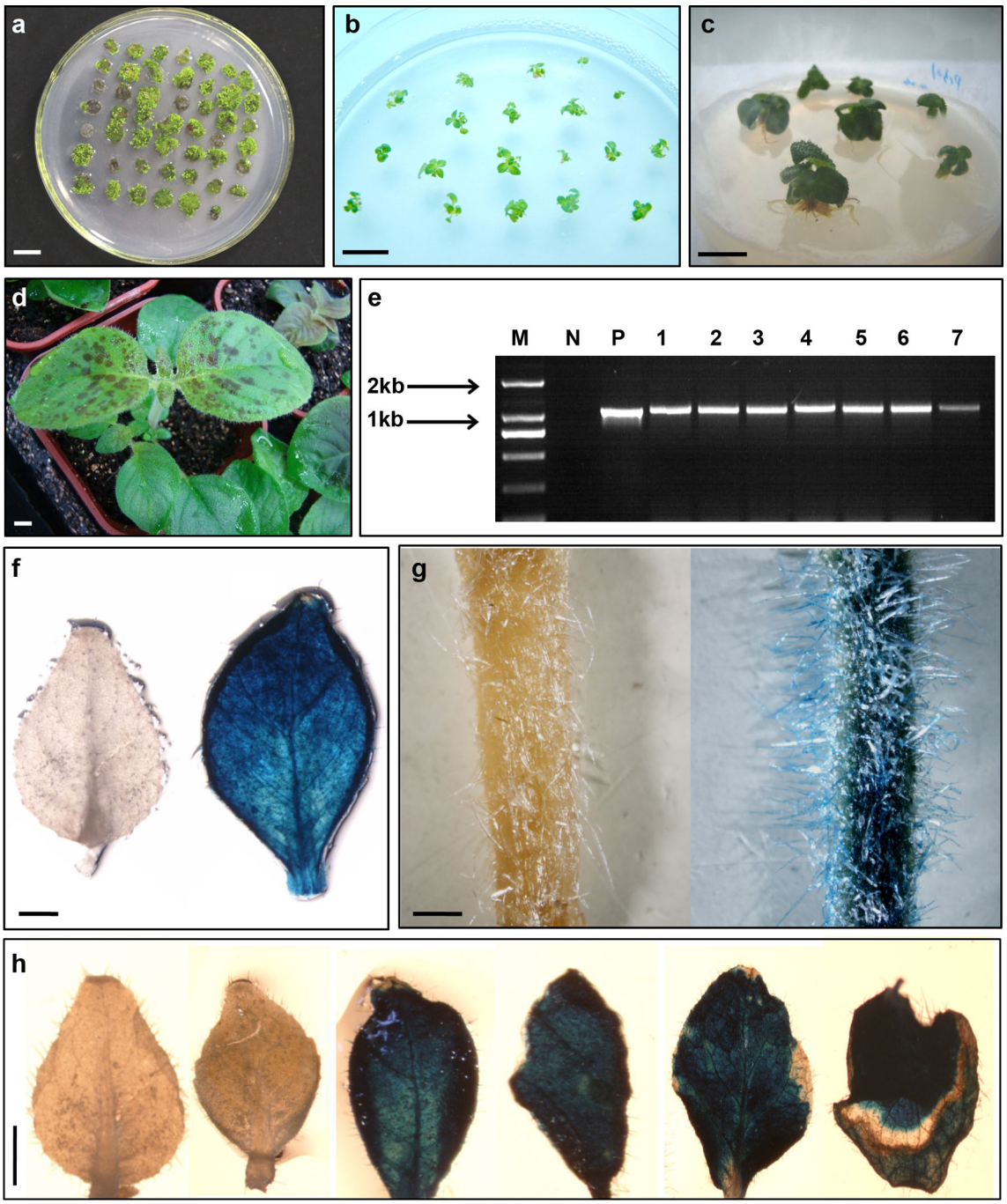

Fig. 6 Transgenic *C. pumila* plants and GUS activity assays. **a** Adventitious shoots appeared on the wound edges of leaf explants. The photo was taken 4 weeks after *Agrobacterium* inoculation. *Bar*, 1 cm. **b** Hygromycin-resistant shoots were transferred onto fresh MS medium to promote rooting and shoot elongation. *Bar*, 1 cm. **c** Shoots with obvious roots after maintained on MS medium for about 1 week. *Bar*, 1 cm. **d** A transgenic plant (photographed 1 month after transplantation). *Bar*, 1 cm. **e** PCR identification of transgenic plants. M, 2 kb DNA marker; N, negative control; P, plasmid DNA; 1–7, seven independent transgenic plants. **f** GUS staining of wild-type (*left*) and transgenic (*right*) leaves. Bar, 1 mm. **g** GUS staining of wild-type (*left*) and T0 transgenic (*right*) stems. *Bar*, 1 mm. **h** GUS staining of wild-type (lane 1 and 2) and T1 transgenic (lane 3–6) leaves

1-day pre-cultured explants with *Agrobacterium* LBA4404 (harboring pCAMBIA1301) for 20 min, culturing on the co-culture medium for 2 days and selecting on the selection medium containing 20 mg l^{-1} hygromycin. Hygromycin-resistant shoots appeared on the wound edges of explants after about 4 weeks of induction (Fig. 6a). After further 4 weeks, the shoots of about 0.5 cm in length were excised and transferred onto fresh MS medium

(without any growth regulator) to promote rhizogenesis and shoot elongation (Fig. 6b). About 1 week later, the roots could be readily observed (Fig. 6c). Plantlets of 1–2 cm in length with well-developed roots were transplanted into pots. Transgenic plants grew well in culture room with purple spots appearing slowly on the old leaves (Fig. 6d). Furthermore, the transgenic plants overexpressing the *GUS* gene were morphologically normal comparing with wild-type ones (Supplementary Fig. 4).

Polymerase chain reaction was carried out to investigate whether the *GUS* gene had been introduced into *C. pumila*. As a result, specific gene products of expected size (1,081 bp) were amplified from seven independent transgenic plants with no fragment amplified from wild-type ones (Fig. 6e). GUS activity assays were conducted to investigate the *GUS* expression. Both leaves and stems of transgenic plants were analyzed with wild-type ones served as negative controls to eliminate the possibility of endogenous GUS expression. The results showed that strong and uniform GUS signal was observed in both leaves and stems of transgenic plants with no expression signal in wild-type ones (Fig. 6f, g).

Progenies of line 2 (Fig. 6e) were further analyzed to validate the *GUS* gene inheritance by culturing on MS selection medium containing 25 mg l^{-1} hygromycin. Of 33 progenies analyzed, 24 could germinate and develop normally, while the remainder slowly became withered after germination, conforming to a Mendelian segregation ratio (3:1) for monogenic inheritance. Subsequent GUS activity assays showed that these hygromycin-resistant seedlings could generate GUS signal with no expression signal in wide-type ones. The above results clearly indicated that the *GUS* gene had been introduced into the host and obtained a stable and uniform expression.

Validation of the transformation system by transferring the *GsNST1B* gene

GsNST1B controlling the secondary wall biosynthesis in *G. soja* (Dong et al. 2013) was introduced into *C. pumila* to validate the availability of this transformation system in gene function investigation. Positive transgenic plantlets were confirmed by PCR followed by DNA sequencing to avoid the possible amplification of endogenous *NST*-like genes. The results showed that of 199 hygromycin-resistant plantlets induced from 55 different leaf explants (for each explant, 3–4 plants were analyzed by PCR), 23 belonging to 6 different transgenic lines were confirmed to be positive. In contrast to wild-type plants that had normally developed leaves (Fig. 7a), transgenic plants showed upward curling leaves (Fig. 7b–d). Sections of the transgenic and wild-type leaves were stained with toluidine blue to understand the cellular basis of upward curling leaves. In wild-type leaves, the secondarily thickened cells were only found in the veins and xylem strands (Fig. 7e). In contrast, the parenchyma mesophyll cells, in addition to the veins and xylem strand cells were heavily secondarily thickened in the transgenic leaves (Fig. 7f). RT-PCR was further carried out to check the expression of the *GsNST1B* gene in transgenic plants. As shown in Fig. 7g, the *GsNST1B* gene was strongly expressed in three independent transgenic plants with no signal in wild-type ones. In addition, *GsNST1B* overexpressors generated undeveloped fruits (data not shown), similar to our previous report (Dong et al. 2013).

Discussion

Characterization of *C. pumila* as an ideal model plant for evo-devo studies

The haploid chromosome number n = 4 of *C. pumila* was previously reported by Ratter (1963). We here document its karyotype 2n = 8 = 6 m + 2sm (2sat), the lowest number of chromosomes reported in Gesneriaceae to date (Skog 1984; Li and Wang 2004; Weber 2004; this study). The species in Gesneriaceae are usually polyploidy with perennial habit (Skog 1984; Li and Wang 2004; Weber 2004). The genus *Chirita* sensu stricto is one of the rarely occurred diploid taxa with annual habit in Gesneriaceae (the traditional polyphyletic *Chirita* was split into four monophyletic groups including the perennial *Primulina* and *Liebigia* and the annual *Chirita* sensu stricto and *Microchirita*; see Wang et al. 2011). The close relatives of *C. pumila* in *Chirita* usually have diploid chromosome number of 2n = 18 (Li and Wang 2004). Given that the basic chromosome number is x = 9 or 8 in Gesneriaceae, the low chromosome number of *C. pumila* was assumed to be achieved through successive unequal translocation from a complement of chromosomes with x = 9, probably correlated with its short-lived habit (Ratter 1963; Skog 1984; Li and Wang 2004). Researches addressing chromosome evolution between *C. pumila* and its relatives would reveal how the rearranged chromosomes contribute to plant habit shifts, reproductive isolation and speciation.

In addition, we found a special phenomenon that *C. pumila* flowers perform self-fertilization before anthesis, i.e. cleistogamy, an extreme form of self-fertilization first reported in Gesneriaceae. Selfing has commonly been viewed as an "evolutionary dead end" because it usually leads to inbreeding depression by accumulating recessive deleterious alleles (Stebbins 1957; Barrett 2002; Boggs et al. 2009). However, when environments become fluctuant and unpredictable with scarcity or inconsistency of pollinators or population bottlenecks, selfing rates would increase with inbreeding depression gradually overcome

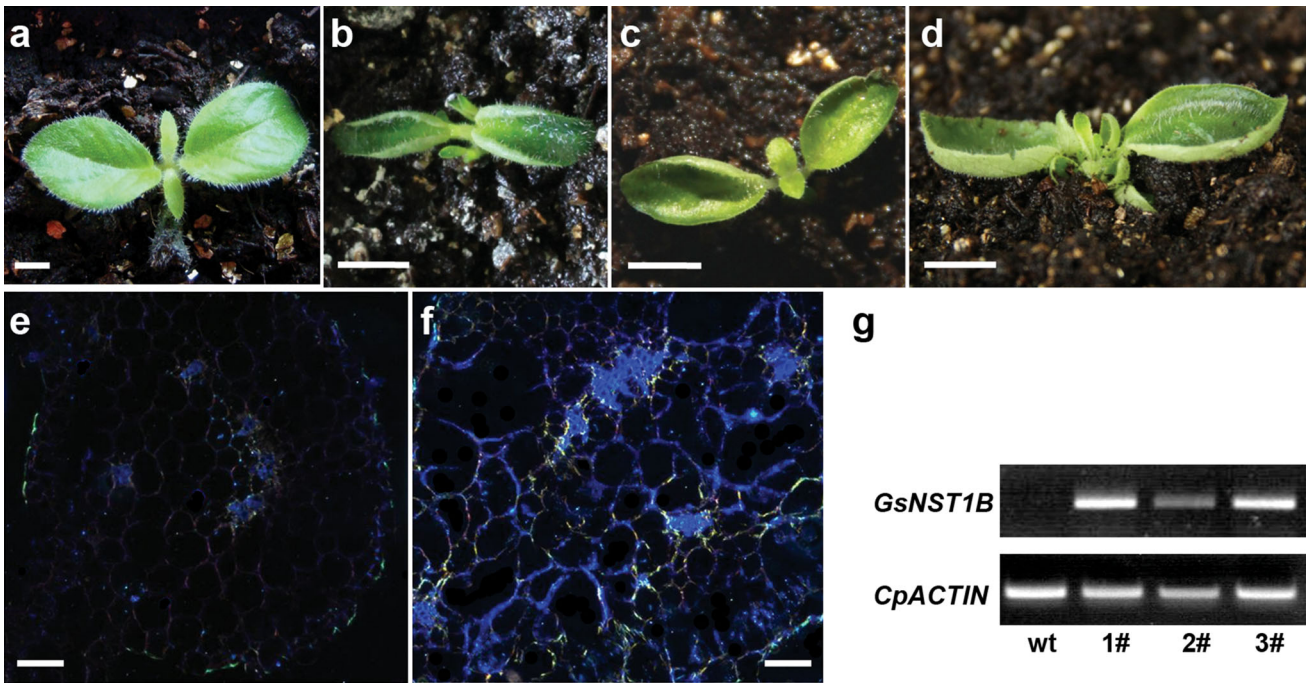

Fig. 7 Analysis of transgenic plants overexpressing *GsNST1B*. **a–d** One wild-type plant **(a)** and three independent transgenic plants **(b–d)** with upward curling leaves. *Bars*, 1 cm**. e** Toluidine *blue* staining results showing normal secondary wall thickening of wild-type plants in the veins and vascular bundles. *Bar*, 10 μm. **f** The ectopic secondary wall thickening of transgenic plants in parenchyma mesophyll cells. *Bar*, 10 μm. **g** RT-PCR analysis of *GsNST1B* in three independent transgenic plants with wild-type plant served as a negative control. *CpACTIN* was amplified as an internal reference

because deleterious alleles may be purged over successive generations of selfing (Barrett 2002; Boggs et al. 2009; Albert et al. 2011). The self-fertilization will finally become established owing to adaptive advantages of self-pollination in providing reproductive assurance when outcrossing fails (Darwin 1876). Morphologically, the conversion from outcrossing to cleistogamy involves the shifts from dichogamy to homogamy and from herkogamy to plesiogamy, and precocious maturation of sexual organs (Campbell et al. 1983). In Gesneriaceae, almost all species have hermaphroditic flowers containing both female and male sexual organs which usually spatially separate in a flower, i.e. herkogamy for cross-pollination. Sexually mature pollen and stigmas are presented as flowers bloom to attract animal pollinators (Wang et al. 2010, 2011). In the evolutionary transition from cross-fertilization to cleistogamy, a series of floral morphological and physiological modifications have occurred in *C. pumila*, including the same position of the anthers and stigmas just below the upper inner surface of the corolla tube, and the precocious and simultaneous maturation of pollen and stigmas before anthesis. In addition, the flowers of *C. pumila* open with anthers included and stigma exserted. Given hand-pollinated flowers producing fertile fruits, *C. pumila* should have the possibility of cross-fertilization, a mixed mating system envisaged as a "bet-hedging strategy" for fluctuating and unpredictable environments (Berg and Redbo-Torstensson 1998; Culley and Klooster 2007). The typical cleistogamous flowers with potential cross-pollination make *C. pumila* an ideal candidate model to understand the ecological success of natural selection and genetic mechanisms for the mating systems of cleistogamy versus chasmogamy.

The ABCE model is a widely used framework to understand the floral development and evolution in angiosperms (Soltis et al. 2007; Litt and Kramer 2010). However, some components of the ABCE model, such as A-function floral identity genes, are so far limited to *Arabidopsis* and its close relatives and their functions have not yet been testified in other lineages of angiosperms (e.g. *Antirrhinum*; Litt 2007; Bowman et al. 2012). Additional function analyses in emerging evo-devo model organisms are therefore critically important to finally elucidate whether the BC model lacking the A-function is general in eudicots (Litt 2007; Soltis et al. 2007; Causier et al. 2010; Bowman et al. 2012). In addition, the origin of zygomorphic flowers is suggested to be one key innovation associated with the explosive radiation of angiosperms (Dilcher 2000; Cubas 2004; Busch and Zachgo 2009). Increasing evidence indicates that *CYC*-like TCP genes play a crucial role in the origin and evolution of floral zygomorphy in angiosperms (Cubas 2004; Busch and Zachgo 2009;

Preston and Hileman 2009; Specht and Bartlett 2009). A recent report suggests that the repeated origins of floral zygomorphy are related to the independent gains of similar positive autoregulatory elements in *CYC*-like TCP genes in different lineages of angiosperms (Yang et al. 2012). However, it is still a challenge to decipher how these genes' activities are controlled by upstream factors, including the dorsal identity function, the functional domain expansion to lateral or ventral floral regions, the loss-of-function and so on (Song et al. 2009; Martín-Trillo and Cubas 2010; Yang et al. 2012; Hileman 2014). *C. pumila* is apparently an ideal model species to address these questions because of its phylogenetic representativeness, annual habit, short life cycle, and self-fertility and diploid, the widely accepted selection criteria for evo-devo model organisms (Irish and Benfey 2004; Jenner 2006; Jenner and Wills 2007; Sommer 2009; Ankeny and Leonelli 2011). Its diploid with low chromosome number would facilitate the identification of recessive traits and avoid the complication of gene dosages, and its typical cleistogamous flowers with potential cross-pollination enable *C. pumila* to be maintained with homozygous lines straightforward and capable of generating genetic crosses. Therefore, we here select *C. pumila* as a target to develop the genetic transformation system.

Efficient *Agrobacterium*-mediated transformation and regeneration of *C. pumila*

An efficient *Agrobacterium*-mediated transformation and regeneration system is developed in *C. pumila* in this study, which depends on a powerful shoot induction ability of leaf explants on MS medium containing 0.5 mg l^{-1} BA and 0.1 mg l^{-1} NAA (Table 1). Moreover, similar to *Perilla frutescens* (Kim et al. 2004), the adventitious shoots of *C. pumila* directly form on the wound edges of leaf explants without an evident callus phase. In addition, *C. pumila* has a powerful rooting ability because no growth regulator is required and roots can be readily observed after 1 week of culture on fresh MS medium. Therefore, *C. pumila* has a powerful regeneration ability using leaf explants, a prerequisite for genetic transformation.

High efficient gene transfer and powerful regeneration ability of explants after *Agrobacterium* inoculation are crucial to plant transformation (He et al. 2010). Many factors may affect the transformation frequency and thereafter the shoot induction rate to varying degrees. As a pivotal step in transformation process, co-culture of the inoculated explants with *Agrobacterium* allows T-DNA transfer from plasmid into plant cells. In general, co-culture for 2–3 days reaches the highest transformation frequency (Kim et al. 2004; Jian et al. 2009). However, co-culture duration can be prolonged to 4–5 days for some species (Mondal et al. 2001; Barik et al. 2005). Here, 2 days of co-culture reaches the highest shoot induction rate. While 1 day of co-culture is insufficient for *Agrobacterium* infection and T-DNA transfer, extended duration may cause the damage of explants owing to the uncontrollable overgrowth of *Agrobacterium*. Accordingly, the shoot induction frequency is reduced in both cases.

It is reported that pre-culture of explants prior to *Agrobacterium* inoculation can significantly enhance the transformation frequency in *Cajanus cajan* (Lawrence and Koundal 2000), *Lathyrus sativus* (Barik et al. 2005), *Lotus corniculatus* (Jian et al. 2009) and *Fagopyrum esculentum* (Chen et al. 2008). However, pre-culture drastically declines the transformation competence of *Citrus paradise* (Costa et al. 2002) and *Perilla frutescens* (Kim et al. 2004). In this study, pre-culture enhances drastically the shoot induction rate, indicating a positive effect of pre-culture on *C. pumila* transformation probably due to the improved viability of explants. However, extended pre-culture reduces slightly the shoot induction rate probably by diminishing the susceptibility of explants to *Agrobacterium*, indicating that only appropriate pre-culture duration is benefit for the transformation and regeneration of *C. pumila*.

Agrobacterium cell density and inoculation time can also affect transformation efficiency (Du and Pijut 2009; Jian et al. 2009). While low *Agrobacterium* cell concentration and short infection time may result in insufficient attachment of *Agrobacterium* to explants and reduce the transformation frequency, increased *Agrobacterium* cells and prolonged inoculation time would damage explants and decrease the regeneration frequency. Here, the highest shoot induction frequency is obtained when fresh leaf explants are infected with *Agrobacterium* cells of $OD_{600} = 0.6$ for 20 min. Both high and low *Agrobacterium* cell density, as well as shortened or prolonged inoculation time reduce the shoot induction rate, indicating that appropriate *Agrobacterium* cell density and inoculation time are important for successful transformation.

As outlined above, a high shoot induction frequency is achieved in *C. pumila* by infecting 1-day pre-cultured explants with *Agrobacterium* of $OD_{600} = 0.6$ for 20 min followed by 2-days of co-culture. Further PCR and GUS activity assays of T0 and T1 plants indicate that the *GUS* gene has been successfully introduced into the host, evident by its stable and uniform expression. However, further transformation experiment using a gene with known function and obvious phenotypic effects is required to validate the applicability of a transformation system in gene function investigation (Jian et al. 2009). Here, the *GsNST1B* gene implicated in secondary wall biosynthesis in *G. soja* is selected due to its obvious phenotype during early vegetative growth stages in *Arabidopsis* overexpressors (Dong et al. 2013). Similar to Dong et al. (2013),

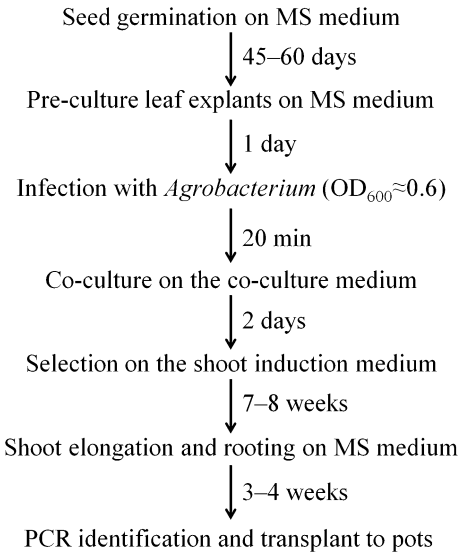

Fig. 8 A flowchart for *Agrobacterium*-mediated transformation of *C. pumila*

GsNST1B overexpression in *C. pumila* generates a desired phenotype characteristic of upward curling rosette leaves that is attributed to the ectopic thickening of secondary walls, indicating the applicability of this transformation system in gene function investigation.

A reliable and efficient transformation system is crucial to comparative functional studies in evo-devo that aims at exploring the evolutionary mechanisms underlying morphological changes. However, developing a transgenic system is usually time-consuming and laborious because it requires several generations of subculture and alterations of medium. In *Lotus japonicas* and *Medicago truncatula*, for example, about 4 months are needed to produce transgenic plants (Stiller et al. 1997; Crane et al. 2006). In *Lotus corniculatus*, the superroot- derived transformation protocol is complicated with at least five changes of medium (Jian et al. 2009). In *Triticum turgidum*, obtaining transgenic lines requires 2–3 rounds of selection (He et al. 2010). As a classical model plant, *Antirrhinum* has been proved to be successful in *Agrobacterium*-mediated genetic transformation accompanied by repeatedly improved transformation protocol (Cui et al. 2003, 2004; Manchado-Rojo et al. 2012). Nevertheless, it still leaves something to be desired that might restrict its wide application. In this study, using leaf disks as explants, the transformation process (from *Agrobacterium* inoculation to PCR identification) takes about 3 months with a high efficiency (Fig. 8). In addition, the entire transformation process is simple because no specific rooting media is required and the shoot induction and selection are achieved in one step. Taken together, the *C. pumila* transformation system has the features of simplicity, rapidity and high-efficiency.

Since its inception at the end of last century, evo-devo has passed from an initial stage to a rapid developing discipline, evident by emerging model organisms in both animals and plants. With completion of genome sequencing project, establishment of a mutant library and further optimization of the transformation system, *C. pumila* could become a fascinating model plant for a wide range of evo-devo studies, especially in the field of floral symmetry, floral organ identity, chromosome evolution and mating system evolution.

Acknowledgments We thank James F. Smith for his constructive comments and language improvements on this article. This work was supported by the National Natural Science Foundation of China (30990240 and 31170198).

References

Albert LP, Campbell LG, Whitney KD (2011) Beyond simple reproductive assurance: cleistogamy allows adaptive plastic responses to pollen limitation. Int J Plant Sci 172(7):862–869

Ankeny RA, Leonelli S (2011) What's so special about model organisms? Stud Hist Philos Sci 42:313–323

Baguñà J, Garcia-Fernàndez J (2003) Evo-devo: the long and winding road. Int J Dev Biol 47:705–713

Barik DP, Mohapatra U, Chand PK (2005) Transgenic grasspea (*Lathyrus sativus* L.): factors influencing *Agrobacterium*-mediated transformation and regeneration. Plant Cell Rep 24:523–531

Barrett SCH (2002) The evolution of plant sexual diversity. Nat Rev Genet 3:274–284

Berg H, Redbo-Torstensson P (1998) Cleistogamy as a bet-hedging strategy in *Oxalis acetosella*, a perennial herb. J Ecol 86:491–500

Bliss BJ, Wanke S, Barakat A et al (2013) Characterization of the basal angiosperm *Aristolochia fimbriata*: a potential experimental system for genetic studies. BMC Plant Biol 13:13

Boggs NA, Nasrallah JB, Nasrallah ME (2009) Independent *S*-locus mutations caused self-fertility in *Arabidopsis thaliana*. PLoS Genet 5(3):e1000426. doi:10.1371/journal.pgen.1000426

Bowman JL, Smyth DR, Meyerowitz EM (2012) The ABC model of flower development: then and now. Development 139(22):4095–4098

Bredemeijer GMM (1982) Mechanism of peroxidase isoenzyme induction in pollinated *Nicotiana alata* styles. Theor Appl Genet 62:305–309

Breuker CJ, Debat V, Klingenberg CP (2006) Functional evo-devo. Trends Ecol Evol 21:488–492

Burr B (2002) Mapping and sequencing the rice genome. Plant Cell 14:521–523

Busch A, Zachgo S (2009) Flower symmetry evolution: towards understanding the abominable mystery of angiosperm radiation. BioEssays 31:1181–1190

Campbell CS, Quinn JA, Cheplick GP, Bell TJ (1983) Cleistogamy in grasses. Annu Rev Ecol Syst 14:411–441

Carroll SB (2008) Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. Cell 134:25–36

Causier B, Schwarz-Sommer Z, Davies B (2010) Floral organ identity: 20 years of ABCs. Semin Cell Dev Biol 21:73–79

Chen L-H, Zhang B, Xu Z-Q (2008) Salt tolerance conferred by overexpression of *Arabidopsis* vacuolar Na^+/H^+ antiporter gene *AtNHX1* in common buckwheat (*Fagopyrum esculentum*). Transgenic Res 17:121–132

Costa MGC, Otoni WC, Moore GA (2002) An evaluation of factors affecting the efficiency of *Agrobacterium*-mediated transformation of *Citrus paradise* (Macf.) and production of transgenic plants containing carotenoid biosynthetic genes. Plant Cell Rep 21:365–373

Crane C, Wright E, Dixon RA, Wang Z-Y (2006) Transgenic *Medicago truncatula* plants obtained from *Agrobacterium tumefaciens*-transformed roots and *Agrobacterium rhizogenes*-transformed hairy roots. Planta 223:1344–1354

Cubas P (2004) Floral zygomorphy, the recurring evolution of a successful trait. BioEssays 26:1175–1184

Cui M-L, Handa T, Ezura H (2003) An improved protocol for *Agrobacterium*-mediated transformation of *Antirrhinum majus* L. Mol Genet Genomics 270:296–302

Cui M-L, Ezura H, Nishimura S, Kamada H, Handa T (2004) A rapid *Agrobacterium*-mediated transformation of *Antirrhinum majus* L. by using direct shoot regeneration from hypocotyl explants. Plant Sci 166:873–879

Culley TM, Klooster MR (2007) The cleistogamous breeding system: a review of its frequency, evolution, and ecology in angiosperms. Bot Rev 73:1–30

Darwin CR (1876) The effects of cross and self-fertilization in the vegetable kingdom, Ch 9. John Murray, London

de Bruijn S, Angenent GC, Kaufmann K (2012) Plant 'evo-devo' goes genomic: from candidate genes to regulatory networks. Trends Plant Sci 17:441–447

Dilcher D (2000) Toward a new synthesis: major evolutionary trends in the angiosperm fossil record. Proc Natl Acad Sci USA 97: 7030–7036

Doležel J, Greilhuber J, Suda J (2007) Estimation of nuclear DNA content in plants using flow cytometry. Nat Protoc 2:2233–2244

Dong Y, Wang B-H, Wang Y-Z (2013) Functional characterization of the orthologs of *AtNST1/2* in *Glycine soja* (Fabaceae) and the evolutionary implications. J Syst Evol 51(6):693–703

Dong Y, Yang X, Liu J, Wang B-H, Liu B-L, Wang Y-Z (2014) Pod shattering resistance associated with domestication is mediated by a NAC gene in soybean. Nat Commun 5:3352

Donoghue MJ, Ree RH, Baum DA (1998) Phylogeny and the evolution of flower symmetry in the Asteridae. Trends Plant Sci 3:311–317

Du N, Pijut PM (2009) *Agrobacterium*-mediated transformation of *Fraxinus pennsylvanica* hypocotyls and plant regeneration. Plant Cell Rep 28:915–923

Du Z-Y, Wang Y-Z (2008) Significance of RT-PCR expression patterns of *CYC*-like genes in *Oreocharis benthamii* (Gesneriaceae). J Syst Evol 46:23–31

Endress PK (1998) *Antirrhinum* and Asteridae—evolutionary changes of floral symmetry. Symp Soc Exp Biol 53:133–140

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049–1051

Gao Q, Tao J-H, Yan D, Wang Y-Z, Li Z-Y (2008) Expression differentiation of *CYC*-like floral symmetry genes correlated with their protein sequence divergence in *Chirita heterotricha* (Gesneriaceae). Dev Genes Evol 218:341–351

He Y, Jones HD, Chen S, Chen XM, Wang DW, Li KX, Wang DS, Xia LQ (2010) *Agrobacterium*-mediated transformation of durum wheat (*Triticum turgidum* L. var. *durum* cv Stewart) with improved efficiency. J Exp Bot 61:1567–1581

Hileman LC (2014) Bilateral flower symmetry—how, when and why? Curr Opin Plant Biol 17:146–152

Irish VF, Benfey PN (2004) Beyond *Arabidopsis*. Translational biology meets evolutionary developmental biology. Plant Physiol 135:611–614

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jeffery WR (2008) Emerging model systems in evo-devo: cavefish and microevolution of development. Evol Dev 10:265–272

Jenner RA (2006) Unburdening evo-devo: ancestral attractions, model organisms, and basal baloney. Dev Genes Evol 216:385–394

Jenner RA, Wills MA (2007) The choice of model organisms in evo-devo. Nat Rev Genet 8(4):311–319

Jian B, Hou W, Wu C, Liu B, Liu W, Song S, Bi Y, Han T (2009) *Agrobacterium rhizogenes*-mediated transformation of *Superroot*-derived *Lotus corniculatus* plants: a valuable tool for functional genomics. BMC Plant Biol 9:78

Jiang LX, Yang SL, Xie LF, San Puah C, Zhang XQ, Yang WC, Sundaresan V, Ye D (2005) *VANGUARD1* encodes a pectin methyl esterase that enhances pollen tube growth in the *Arabidopsis* style and transmitting tract. Plant Cell 17:584–596

Kellogg EA (2004) Evolution of developmental traits. Curr Opin Plant Biol 7:92–98

Kellogg EA (2006) Progress and challenges in studies of the evolution of development. J Exp Bot 57:3505–3516

Kim K-H, Lee Y-H, Kim D, Park Y-H, Lee J-Y, Hwang Y-S, Kim Y-H (2004) *Agrobacterium*-mediated genetic transformation of *Perilla frutescens*. Plant Cell Rep 23:386–390

Kopp A (2009) Metamodels and phylogenetic replication: a systematic approach to the evolution of developmental pathways. Evolution 63:2771–2789

Kramer EM (2007) Understanding the genetic basis of floral diversity. Bioscience 57:479–487

Kushikawa S, Hoshino Y, Mii M (2001) *Agrobacterium*-mediated transformation of *Saintpaulia ionantha* Wendl. Plant Sci 161:953–960

Lawrence PK, Koundal KR (2000) Simple protocol for *Agrobacterium tumefaciens*-mediated transformation of pigeon pea [*Cajanus cajan* (L.) Millsp.]. J Plant Biol 27:299–302

Li Z-Y, Wang Y-Z (2004) Plants of Gesneriaceae in China. Henan Science and Technology Publishing House, Zhengzhou

Litt A (2007) An evaluation of A-function: evidence from the APETALA1 and APETALA2 gene lineages. Int J Plant Sci 168:73–91

Litt A, Kramer EM (2010) The ABC model and the diversification of floral organ identity. Semin Cell Dev Biol 21:129–137

Liu B-L, Pang H-B, Yang X, Wang Y-Z (2014) Functional and evolutionary analyses of *Primulina heterotricha CYC1C* gene in tobacco and *Arabidopsis* transformation systems. J Syst Evol 52(1):112–123

Manchado-Rojo M, Delgado-Benarroch L, Roca MJ, Weiss J, Egea-Cortines M (2012) Quantitative levels of *Deficiens* and *Globosa* during late petal development show a complex transcriptional network topology of B function. Plant J 72:294–307

Mandoli DF, Olmstead R (2000) The importance of emerging model systems in plant biology. J Plant Growth Regul 19:249–252

Martín-Trillo M, Cubas P (2010) TCP genes: a family snapshot ten years later. Trends Plant Sci 15:31–39

Mercuri A, De Benedetti L, Burchi G, Schiva T (2000) *Agrobacterium*-mediated transformation of African violet. Plant Cell Tissue Organ Cult 60:39–46

Mondal TK, Bhattacharya A, Ahuja PS, Chand PK (2001) Transgenic tea [*Camellia sinensis* (L.) O Kuntze cv. Kangrajat] plants obtained by *Agrobacterium*-mediated transformation of somatic embryos. Plant Cell Rep 20:712–720

Mori T, Karoiwa H, Higashiyama T, Kuroiwa T (2006) GENERATIVE CELL SPECIFIC 1 is essential for angiosperm fertilization. Nat Cell Biol 8:63–71

Müller GB (2007) Evo-devo: extending the evolutionary synthesis. Nat Rev Genet 8:943–949

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15:473

Pang H-B, Sun Q-W, He S-Z, Wang Y-Z (2010) Expression pattern of *CYC*-like genes relating to a dorsalized actinomorphic flower in *Tengia* (Gesneriaceae). J Syst Evol 48:309–317

Preston JC, Hileman LC (2009) Developmental genetics of floral symmetry evolution. Trends Plant Sci 14:147–154

Pruitt RE, Bowman JL, Grossniklaus U (2003) Plant genetics: a decade of integration. Nat Genet Suppl 33:294–304

Ratter JA (1963) Some chromosome members in the Gesneriaceae. Notes from the Royal Botanic Garden, Edinburgh

Skog LE (1984) A review of chromosome numbers in the Gesneriaceae. Selbyana 7:252–273

Soltis DE, Ma H, Frohlich MW et al (2007) The floral genome: an evolutionary history of gene duplication and shifting patterns of gene expression. Trends Plant Sci 12:358–367

Sommer RJ (2009) The future of evo-devo: model systems and evolutionary theory. Nat Rev Genet 10:416–422

Song C-F, Lin Q-B, Liang R-H, Wang Y-Z (2009) Expressions of ECE-CYC2 clade genes relating to abortion of both dorsal and ventral stamens in *Opithandra* (Gesneriaceae). BMC Evol Biol 9:244

Specht CD, Bartlett ME (2009) Flower evolution: the origin and subsequent diversification of the angiosperm flower. Annu Rev Ecol Evol Syst 40:217–243

Stebbins GL (1957) Self-fertilization and population variability in the higher plants. Am Nat 91:337–354

Stern DL (2000) Perspective: evolutionary developmental biology and the problem of variation. Evolution 54:1079–1091

Stiller J, Martirani L, Tuppale S, Chian R-J, Chiurazzi M, Gresshoff PM (1997) High frequency transformation and regeneration of transgenic plants in the model legume *Lotus japonicus*. J Exp Bot 48:1357–1365

Tang Z, Lin H, Shi L, Chen W (2007a) Rapid *in vitro* multiplication of *Chirita longgangensis* W. T. Wang: an endemic and endangered Gesneriaceae species in China. HortScience 42:638–641

Tang Z, Shi L, Chen W, Lin H (2007b) *In vitro* propagation of *Chirita heterotricha* Merr. Propag Ornam Plants 7:43–48

Tóth S, Kiss C, Scott P, Kovács G, Sorvari S, Toldi O (2006) *Agrobacterium*-mediated genetic transformation of the desiccation tolerant resurrection plant *Ramonda myconi* (L.) Rchb. Plant Cell Rep 25:442–449

Wang W, Pan K, Li Z, Weitzman AL, Skog LE (1998) Gesneriaceae. Flora China 18:244–401

Wang Y-Z, Liang R-H, Wang B-H, Li J-M, Qiu Z-J, Li Z-Y, Weber A (2010) Origin and phylogenetic relationships of the Old World Gesneriaceae with actinomorphic flowers inferred from ITS and *trnL-trnF* sequences. Taxon 59(4):1044–1052

Wang Y-Z, Mao R-B, Liu Y, Li J-M, Dong Y, Li Z-Y, Smith JF (2011) Phylogenetic reconstruction of *Chirita* and allies (Gesneriaceae) with taxonomic treatments. J Syst Evol 49:50–64

Weber A (2004) Gesneriaceae. In: Kubitzki K, Kadereit JW (eds) The families and genera of vascular plants. Volume VII, flowering plants: Dicodyledons; Lamiales (except Acanthaceae including *Avicenniaceae*). Springer, Berlin, pp 63–158

Wortley AH, Rudall PJ, Harris DJ, Scotland RW (2005) How much data are needed to resolve a difficult phylogeny? Case study in Lamiales. Syst Biol 54:697–709

Yang X, Cui H, Yuan Z-L, Wang Y-Z (2010) Significance of consensus CYC-binding sites found in the promoters of both *ChCYC* and *ChRAD* genes in *Chirita heterotricha* (Gesneriaceae). J Syst Evol 48:249–256

Yang X, Pang H-B, Liu B-L, Qiu Z-J, Gao Q, Wei L, Dong Y, Wang Y-Z (2012) Evolution of double positive autoregulatory feedback loops in CYCLOIDEA2 clade genes is associated with the origin of floral zygomorphy. Plant Cell 24:1834–1847

Zhang MZ, Ye D, Wang LL, Pang JL, Zhang YH, Zheng K, Bian HW, Han N, Pan JW, Wang JH, Zhu MY (2008) Overexpression of the cucumber LEAFY homolog CFL and hormone treatments alter flower development in gloxinia (*Sinningia speciosa*). Plant Mol Biol 67:419–427

Zhou X-R, Wang Y-Z, Smith JF, Chen RJ (2008) Altered expression patterns of TCP and MYB genes relating to the floral developmental transition from initial zygomorphy to actinomorphy in *Bournea* (Gesneriaceae). New Phytol 178:532–543