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ORIGINAL PAPER

Genetic transformation and overexpression of a rice *Hd3a* induces early flowering in *Saussurea involucrata* Kar. et Kir. ex Maxim

Meiru Li · Hongqing Li · Xiaoying Hu · Xiaoping Pan · Guojiang Wu

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Abstract *Saussurea involucrata* is a valuable traditional Chinese medicinal herb. This is the first report of a successful genetic transformation protocol for *S. involucrata* using *Agrobacterium tumefaciens*. Leaf explants were incubated with *A. tumefaciens* strain EHA105 harboring the binary vector pCAMBIA 1301, which contains the *hpt* gene as a selectable marker for hygromycin resistance and an intron-containing *β*-glucuronidase gene as a reporter gene. Following co-cultivation, about 23.7% of the explants produced hygromycin-resistant calli on MS basal medium (Murashige and Skoog in Physiol Plant 15: 473–497, 1962) supplemented with 1 mg l^{-1} benzyladenine (BA), 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA), 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 20 mg l^{-1} hygromycin, and 500 mg l^{-1} cefotaxime. Shoots were regenerated following transfer of the resistant calli to shoot induction medium containing 1.5 mg l^{-1} BA, 0.1 mg l^{-1} NAA, 0.25 mg l^{-1} gibberellic acid (GA3), 20 mg l^{-1} hygromycin, and 250 mg l^{-1} cefotaxime, and about 67.5% of the resistant calli differentiated into shoots. Finally, 80% of the hygromycin-resistant shoots rooted on MS media supplemented with 0.2 mg l^{-1} NAA, 20 mg l^{-1} hygromycin, and 250 mg l^{-1} cefotaxime. The transgenic nature of the transformants was demonstrated by detection of *β*-glucuronidase activity in the primary transformants and by Southern blot hybridization analysis. About 16% of the total inoculated leaf explants produced transgenic plants after approximately 5 months. Using this optimized transformation system, a rice ortholog of the *Arabidopsis FLOWERING LOCUS T* gene, *Hd3a*, was transferred into *S. involucrata*. Introduction of this gene caused an early-flowering phenotype in *S. involucrata*.

Keywords *Saussurea involucrata* Kar. et Kir. ex Maxim · Transformation · *Hd3a* · Early flowering

M. Li · X. Hu · X. Pan · G. Wu (✉)
Key Laboratory of Plant Resources Conservation and Sustainable Utilization, South China Botanical Garden, Chinese Academy of Sciences, 510650 Guangzhou, People's Republic of China
e-mail: wugj@scbg.ac.cn

H. Li
Guangdong Provincial Key Lab of Biotechnology for Plant Development, South China Normal University, 510631 Guangzhou, People's Republic of China

Introduction

Saussurea involucrata Kar. et Kir. ex Maxim is a valuable traditional Chinese medicinal herb of the family Asteraceae. The plant has long been used under the herbal name "Tianshan snow lotus" for treatment of rheumatoid arthritis, cough with cold, stomachache, dysmenorrhea, and altitude sickness (Encyclopaedia of Traditional Chinese Medicine 1995) and has recently been found to have anti-inflammatory (He et al. 1990; Jia et al. 2005; Chen et al. 2010), antifatigue (Zheng et al. 1993), antitumor (Wu et al. 2009), and anticancer actions (Liu et al. 1985; Han 1995; Way et al. 2010). *S. involucrata* is a perennial herbaceous plant, a special local plant growing only in the rocky mountains and glaciers near or above the snowline about 4,000 m above sea level in Xinjiang, Tibet, and Yunnan Provinces of China, having the ability to resist harsh climates with severe cold, intense ultraviolet radiation, and low oxygen. The plant begins to blossom around the middle of August in the fifth year of its sexennial growth period. The plant whole height is 14–50 cm, having a dense basal rosette of oblanceolate leaves spiraling up the

flowering stem, and a dense woolly head of capitulum with multilayer pale-greenish-yellow phyllaries and purple florets (Chen et al. 1999). Unfortunately, basic biological studies are limited due to the difficulty of reproductive success of this plant under normal growth conditions and the lack of efficient and rapid transformation protocols.

Owing to overexploitation of wild plants and difficulty of cultivation, *S. involucrata* is now almost extinct in China (Fu 1992). Successful application of plant biotechnology for conservation of rare and endangered plant resources and development of novel germplasm will largely depend on the availability of an efficient plant regeneration and genetic transformation system (Offord and Tyler 2009; Bakhshaie et al. 2010; Irvani et al. 2010; Li et al. 2008a, b). Recently, the potential of biotechnology in *S. involucrata* conservation and exploitation for commercial purposes has been recognized. Several reports have described plant regeneration from embryos (Wu et al. 2005a, b), leaf explants (Yang and Zou 2005; Lin et al. 2006; Guo et al. 2007; Zhao and Wang 2008), and hairy roots (Fu et al. 2004) of *S. involucrata*. Regenerated plants successfully survived and blossomed in the third year after transplanting to Taishan Mountain (Zhao and Wang 2008).

As wild *S. involucrata* grows only in special regions with peculiar climate, and cultivation of the plant in a normal climate has been unsuccessful so far, rapid mass production of *S. involucrata* for medicinal purposes is limited, and the development and genetic background of this plant are also unknown. Flowering time is very important for reproductive success of higher plants. Therefore, we tried to set up a flower regeneration system in *S. involucrata* to facilitate basic biological studies of flower development, reproductive success of this plant in a normal climate, rapid mass production of the flowers for medicinal purposes, and the study of bioactive phytochemicals in the flowers.

FLOWERING LOCUS T (*FT*), a well-known floral integrator gene, plays an important role in controlling flowering time in higher plants (Kardailsky et al. 1999; Kobayashi et al. 1999; Xi and Yu 2009). More recently, *FT* and its orthologs have been proposed as florigens, or mobile flowering signals, migrating from leaves to the apical meristem to promote floral initiation (Tamaki et al. 2007; Corbesier et al. 2007; Lin et al. 2007). Overexpression of *Arabidopsis thaliana FT* or ectopic expression of *FT* orthologs such as *CiFT* of trifoliate orange (Kobayashi et al. 1999), *Hd3a* of *Oryza sativa* L. (Kojima et al. 2002), *PtFT1* of *Populus trichocarpa* (Böhlenius et al. 2006), *FT2* of *Populus deltoids* (Hsu et al. 2006), and *OnFT* of orchid (Hou and Yang 2009) has been shown to cause an early-flowering phenotype in *Arabidopsis*. Furthermore, both *PtFT1* and *FT2* were successfully used to dramatically accelerate flowering in poplar (Böhlenius et al. 2006; Hsu et al. 2006; Zhang et al. 2010), and *CiFT* was also successfully applied to induce early flowering in trifoliate orange (Endo et al. 2005) and European pear (Matsuda et al. 2009).

In this study, an efficient genetic transformation procedure for *S. involucrata* using an *Agrobacterium tumefaciens*-mediated leaf disc transformation method was developed. Based on this transformation protocol, a rice ortholog of the *Arabidopsis FLOWERING LOCUS T* gene, *Hd3a*, was transformed into the genome of *S. involucrata*. Flowering cluster regenerated directly from the callus transformed with *Ubiquitin::Hd3a*.

Materials and methods

Plant materials and tissue culture conditions

Seeds of *Saussurea involucrata* Kar. et Kir. ex Maxim were collected from Xinjiang, P.R. China. The seeds were surface-sterilized as previously described (Li et al. 2008a, b) and then placed on Petri dishes containing half-strength Murashige and Skoog (1962) salt (1/2 MS) solid medium containing 100 mg l^{-1} myo-inositol, 10 mg l^{-1} thiamine-HCl, 3% (w/v) sucrose, and 0.7% (w/v) agar (Sigma) to germinate. Tissue cultures were incubated at 24 ± 1°C with a 14-h photoperiod under illumination at 35 μmol m^{-2} s^{-1} from cool-white fluorescent lamps throughout the experiments, except callus induction, which was conducted under darkness. All plant media were adjusted with 1 N KOH or 1 N HCl to pH 5.8, solidified with 0.7% (w/v) agar (Sigma, St. Louis, MO, USA), and autoclaved at 121°C for 20 min.

Plasmid preparation and bacterial cells

The binary vector pCAMBIA 1301 containing both the reporter gene for β-glucuronidase (*uidA*) and the selectable marker gene for hygromycin phosphotransferase (*hpt*) was used for genetic transformation experiments firstly. A *Hin*dIII-*Bam*HI fragment containing maize *Ubiquitin* promoter with *Ubiquitin* intron from pAHC27 (Takimoto et al. 1994) was cloned into the *Hin*dIII-*Bam*HI sites of the binary vector pCAMBIA1390; this vector was named p1390Ubi. The complete open reading frame (ORF) of *Hd3a* (accession no. Os06g0157700) was amplified from rice leaves by reverse-transcription polymerase chain reaction (RT-PCR) using the following primers: forward 5′-AATGAATTC (*Eco*RI site) ATGGCCGGAAGTGG CAGGGAC-3′ and reverse 5′-AATGAATTC (*Eco*RI site) CTAGGGGTAGACCCTCCTG-3′. The fragment was cloned into the *Eco*RI site of p1390Ubi in the sense orientation (Fig. 1; p1390UbiHd3a). The constructed

p1390UbiHd3a was confirmed by sequencing. Both pCAMBIA 1301 and p1390UbiHd3a were then transformed into *Agrobacterium tumefaciens* strain EHA105 by the liquid-nitrogen freeze–thaw method (Chen et al. 1994), respectively.

For plant genetic transformation, a single colony of the bacteria was inoculated in YEP medium supplemented with kanamycin (50 mg l^{-1}) and rifampicin (50 mg l^{-1}) and cultured overnight at 28°C. Bacterial cells were collected by centrifugation at 10,000*g* for 30 s at 25°C and resuspended in 30 ml (OD_{600} = 0.4–0.5) liquid MS medium (pH 5.2) supplemented with 20 mg l^{-1} acetosyringone (AS). After incubation on a shaker (160 rpm) for 3 h at 28°C, the *Agrobacterium* was used to infect the explants.

Genetic transformation of *S. involucrata*

Leaves from 1-month-old seedlings were cut into 0.5 × 0.5 cm^2 and inoculated with *Agrobacterium* cells harboring pCAMBIA 1301 in 30 ml liquid MS medium supplemented with 20 mg l^{-1} AS for 15 min. Subsequently, the explants were blotted on sterile filter paper to remove most of the liquid medium, and transferred to agar-solidified (0.7% w/v) callus induction medium, which is MS basal medium supplemented with 1 mg l^{-1} benzyladenine (BA), 0.1 mg l^{-1} α-naphthaleneacetic acid (NAA), 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) (Zhao and Wang 2008), and 20 mg l^{-1} AS for co-cultivation. After 3 days, the explants were washed with sterile water containing 500 mg l^{-1} cefotaxime to remove *Agrobacterium* cells. Subsequently, the explants were transferred onto selection medium, which is callus induction medium supplemented with 20 mg l^{-1} hygromycin and 500 mg l^{-1} cefotaxime. Eighteen days later, the hygromycin-resistant calli were transferred onto hygromycin-resistant shoot regeneration medium, which is MS basal medium supplemented with 1.5 mg l^{-1} BA, 0.1 mg l^{-1} NAA, 0.25 mg l^{-1} gibberellic acid (GA3), 20 mg l^{-1} hygromycin, and 250 mg l^{-1} cefotaxime and incubated in the light. Transfer onto the same fresh hygromycin-resistant shoot regeneration media was carried out at 3-week intervals. After another 8 weeks, hygromycin-resistant shoots (4–5 cm in height) were separated and transferred to MS basal medium supplemented with 0.2 mg l^{-1} NAA (Zhao and Wang 2008), 20 mg l^{-1} hygromycin, and 250 mg l^{-1} cefotaxime for rooting. After 4 weeks of growth, the regenerated plants were repotted into plastic pots containing autoclaved vermiculite and sand (1/1, v/v) for further aseptic growth under plant growth chamber conditions (17 ± 1°C in a 14-h photoperiod under illumination at 35 μmol m^{-2} s^{-1} from cool-white fluorescent lamps).

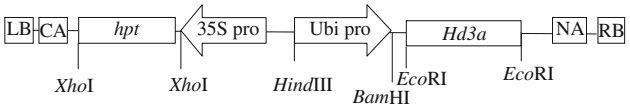

Fig. 1 T-DNA region of the constructed p1390UbiHd3a. The full-length complementary DNA (cDNA) encoding the rice *Hd3a* gene (539 bp) was inserted into the *Eco*RI site of p1390Ubi in the sense orientation. *RB* right border of the T-DNA, *LB* left border of the T-DNA, *35S pro* 35S promoter from cauliflower mosaic virus, *Ubi pro Ubiquitin* promoter with *Ubiquitin* intron from maize, *CA* CaMV35S polyA, *NA* nopaline synthase polyA, *hpt* hygromycin phosphate transferase

For genetic transformation of *Hd3a* into the genome of *S. involucrata*, leaf explants were inoculated with *Agrobacterium* cells harboring p1390UbiHd3a, and the subsequent tissue culture was performed as described above.

Southern and Northern bolt analyses

Genomic DNA was isolated from leaves of nontransformed and transformed plants as well as from transformed flowers using the cetyl trimethylammonium bromide (CTAB) method (Muhammad et al. 1994). Firstly, to examine whether any residual *Agrobacterium* existed in the regenerated plants, *Agrobacterium VirB1* gene-specific primers VBFw (5′-GAA GGC AAC AGG GCC GCT GTC-3′) and VBRe (5′-TCC GCC CTC CGG GGA ACG ACG C-3′) were used to amplify the 630-bp fragment from *VirB1*. The reaction mixture for PCR of *hpt* was incubated in a DNA thermal cycler under the following conditions: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, with a final 10 min extension at 72°C; for PCR of *Hd3a*, the annealing temperature was 55°C. For Southern blot analysis, 5 μg genomic DNA was digested with the restriction enzyme *Hin*dIII, separated on 0.8% (w/v) agarose gel, and transferred to Hybond N^+ nylon membrane. The *hpt* probe was labeled with digoxigenin (DIG) by PCR method using the primers HFw (5′-CGA TCT TAG CCA GAC GAG CGG GTT C-3′) and HRe (5′-GCT GGG GCG TCG GTT TCC ACT ATC GG-3′), while the *Hd3a* probe amplification used the primers Hd3aFw (5′-ATG GCC GGA AGT GGC AGG GAC-3′) and Hd3aRe (5′-CTA GGG GTA GAC CCT CCT G-3′). Prehybridization, washing, and chemiluminescent detection of the blots were performed according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany).

For Northern blot analysis, RNA was extracted from leaves of nontransformed and transformed plants as well as from transformed flowers according to the Trizol method (Invitrogen, Carlsbad, CA, USA), and 10 μg RNA from each line was used for running on a formaldehyde gel and then blotted to a Hybond N^+ membrane. The same *Hd3a* probe was used to detect the transcript of *Hd3a*.

Assay for *uidA* expression in transgenic plantlet

Histochemical staining of β-glucuronidase (GUS) activity was performed according to Jefferson (1987). Hygromycin-resistant callus, transformed buds, and nontransformed buds (wild type) were submerged in a solution containing 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) at 37°C for 3 h and then destained in 70% alcohol.

Results

Establishment of an efficient *Agrobacterium*-mediated leaf disc transformation method

We selected hygromycin as the selection reagent in genetic transformation of *Saussurea involucrata* Kar. et Kir. ex Maxim according to Li et al. (2006), and demonstrated that all leaves turned brown quickly and failed to produce callus in 2 weeks, and that all buds gradually became brown and died in 2–3 weeks under the selection pressure of 20 mg l^{-1} hygromycin (Fig. 2a). The *Agrobacterium* strain EHA105 harboring pCAMBIA1301 vector, which contains the *hpt* gene and *uidA* gene, was used to infect the leaf explants. Highly efficient transient *GUS* expression was observed after 3 days of co-cultivation (Table 1). Hygromycin-resistant callus began to be observed on the surface of the infected brown leaves after 10 days of selection. After 18 days of selection culture, the frequency of resistant calli was about 23.7%. Because of bacterial overgrowth on the brown leaf explants, the calli were divided from the brown explants and transferred to hygromycin-resistant shoot regeneration medium (Fig. 2b). Shoot regeneration began to be observed on the surface of the callus after 35 days of culture, and about 67.5% of the hygromycin-resistant calli could differentiate into shoots (Table 1). Subsequently, all the shoots could further develop on the resistant shoot selected medium (Fig. 2c), and 80% of the hygromycin-resistant shoots rooted on the rooted medium successfully (Fig. 2d).

Histochemical GUS assay showed that the transgene was expressed in resistant calli (Fig. 2e) and resistant buds (Fig. 2f), while there was no GUS staining in buds of nontransformed plant control (Fig. 2g).

Southern blot analysis was further used to confirm the presence of T-DNA in the *S. involucrata* genome. Firstly, the *VirB1*-specific primers were also used to examine the presence of residual *Agrobacterium* in the regenerated buds. Amplification of the genomic DNA from *Agrobacterium* produced the 630-bp band, while amplification of DNAs from different transgenic buds did not give this band (data not shown). Southern blot analysis using the *hpt* probe was performed on randomly selected transgenic lines

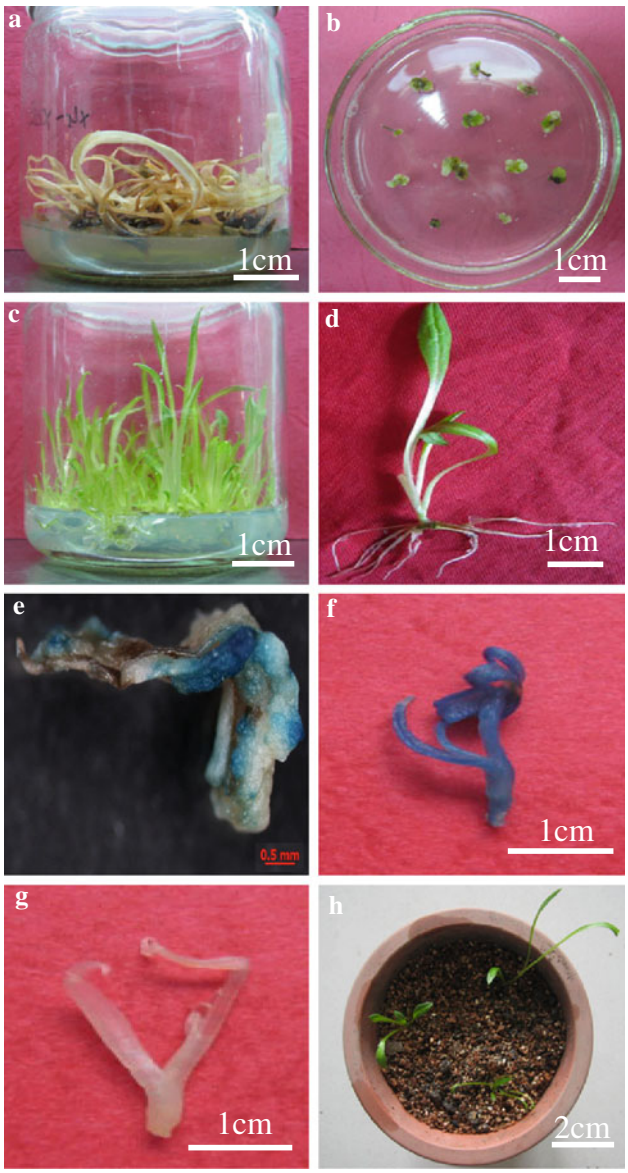

Fig. 2 Regeneration of transgenic plants from leaf explants in *Saussurea involucrata* Kar. et Kir. ex Maxim. **a**, **c** Non-transformed buds (**a**) and transformed buds (**c**) after a 20-day culture on hygromycin-resistant shoot regeneration medium. **b** Hygromycin-resistant calli after a 6-day culture in the light on hygromycin-resistant shoot regeneration MS medium containing 1.5 mg l^{-1} BA, 0.1 mg l^{-1} NAA, 0.25 mg l^{-1} GA3, 20 mg l^{-1} hygromycin, and 250 mg l^{-1} cefotaxime. **d** Regenerated plantlet after a 28-day culture on rooting medium. **e**, **f**, **g** GUS assay of transformed callus derived from leaf explant (**e**), bud (**f**), and non-transformed bud (**g**). **h** Regenerated plants grown in plant growth chamber after a 45-day culture

(T1, T2, T3, and T4). The results showed that all the selected lines had T-DNA integrated in their genome. As the genomic DNA was restricted with *Hin*dIII, which cuts only once inside the T-DNA region in pCAMBIA 1301, the number of hybridization bands reflects the copy number of the T-DNA integrated into the *S. involucrata* genome.

Table 1 Frequency of transient, GUS-positive, and hygromycin-resistant callus as well as hygromycin-resistant buds of *Saussurea involucrata* Kar. et Kir. ex Maxim

Experiment	No. of leaves incubated[a2]	Frequency of transient GUS expression (%)[a1]	No. of hygromycin-resistant calli[b]	No. of hygromycin-resistant buds[c]	GUS+[d]
1	52	80	12	8	8
2	69	90	16	12	11
3	48	80	12	7	7

[a] Ten pieces of leaf were taken for transient GUS expression assay on the third day after infection with *Agrobacterium* in each experiment; [a1]Explants with blue spots were identified and counted using a dissection microscope (Stemi SV 11; Zeiss, Oberkochen, Germany). [a2]Excluding the 10 explant pieces, the number of infected leaves transferred to the hygromycin-resistant callus selection medium was used as the number of leaves incubated

[b] Data were collected after the infected leaf explants were transferred to hygromycin-resistant callus selection medium for 18 days under darkness

[c] Data were collected after resistant calli were transferred to hygromycin-resistant shoot regeneration medium for 8 weeks in the light

[d] Hygromycin-resistant buds positive on GUS staining

The T-DNA copies integrated into the transgenic lines ranged from one to two (Fig. 3). No hybridization signal was detected in samples of nontransformed plants. These results indicated that all the transgenic plants were from independent transgenic events, as they had different copy numbers of T-DNA integrated into different positions of the genome.

Totally, 27 independent transgenic lines were regenerated from 169 leaf explants in three independent experiments. On average, 16 transgenic lines were obtained from every 100 leaf explants after approximately 5 months (Table 1). The rooted transgenic plantlets were repotted in a plant growth chamber at 17 ± 1°C with a 14-h photoperiod under illumination at 35 μmol m^{-2} s^{-1} from cool-white fluorescent lamps; about 75% of the transgenic plants survived under these conditions after 45 days of transplanting (Fig. 2h).

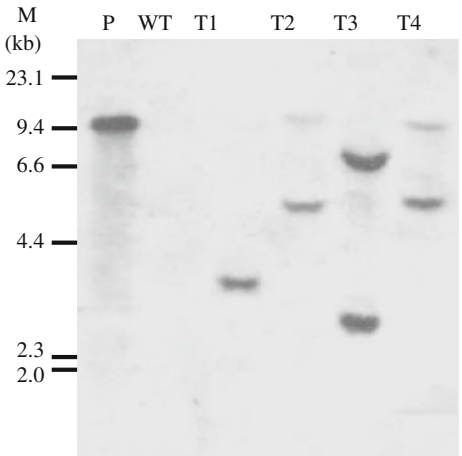

Fig. 3 Southern blot hybridization of the *hpt* gene in transgenic lines. *M*, ladder. *P*, plasmid vector pCAMBIA 1301. *WT*, regenerated nontransformed plants. *T1*, *T2*, *T3*, *T4*, different, randomly selected transformed plants

Ectopic expression of *Hd3a* causes early flowering

To explore whether *Hd3a* from rice is able to induce early flowering in *S. involucrata*, *Hd3a* driven by the maize *Ubiquitin* promoter with *Ubiquitin* intron was transformed into *S. involucrata* genome for functional analysis using the transformation method described above. The first flower-like structure initiated directly from the hygromycin-resistant callus after 12 days on the shoot regeneration medium (Fig. 4a, b), compared with 28 days for regenerated shoot, while in wild-type *S. involucrata*, it takes 5–6 years to blossom in special regions with peculiar climate (Chen et al. 1999). Twenty independent *Ubiquitin::Hd3a* transgenic *S. involucrata* lines were obtained. Fifteen lines showed identical phenotypes, which were flower structures initiated directly from callus without any leaves or buds (Fig. 4c, d, e), one line with obvious stem blossomed in 45 days after transfer onto shoot regeneration medium (Fig. 4f), another line blossomed in 28 days after transfer onto rooting medium (Fig. 4g), whereas three lines were phenotypically indistinguishable from untransformed wild-type plants, one blossoming in 20 days after transplanting to the plant growth chamber (Fig. 4h).

Southern blot analysis was used to confirm the presence of *Hd3a* in the transgenic *S. involucrata* genome, as well as the copy number integrated. As the genomic DNA was restricted with *Hin*dIII, which is the only restriction site in T-DNA region, the number of hybridization bands reflected the copy number of the T-DNA integrated into the *S. involucrata* genome (Fig. 1). Southern blotting using *Hd3a* as a probe detected hybridization signal in nontransformed plants, indicating that *S. involucrata* contains *Hd3a*-homologous sequences. We observed hybridization signals in samples from transgenic lines (lines 1 and 2 are flower clusters; lines 3 and 4 are flowering plants) that differed from those observed in nontransformed samples (Fig. 5a).

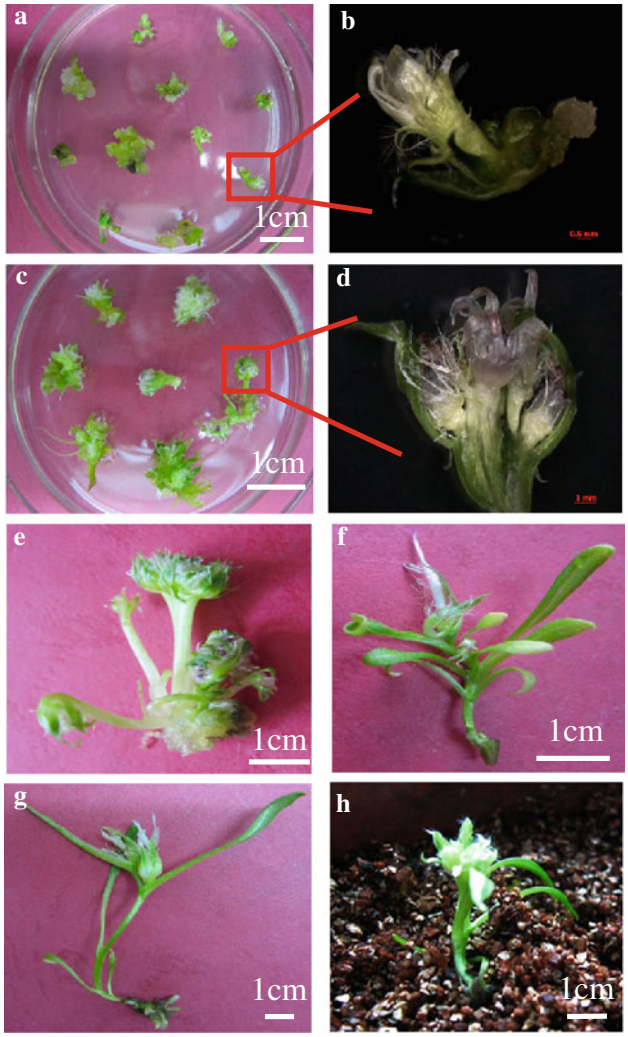

Fig. 4 *Hd3a* overexpression induces early flowering in *Saussurea involucrata* Kar. et Kir. ex Maxim. **a**, **c** Overview of a tissue culture plate with hygromycin-resistant calli transformed with *Ubiquitin::Hd3a* after 18-day culture (**a**) and 28-day culture (**c**) on shoot regeneration medium. **b**, **d** Close-up view of a developing flower initiated directly from callus (**b**) and a section of a flower (**d**). **e** Flowering cluster initiated directly from callus after 35 days on shoot regeneration medium. **f** Flowering stem after 45 days on shoot regeneration medium. **g** Regenerated flowering plant after 28-day culture on rooting medium. **h** Regenerated plant blossomed in plant growth chamber after 20-day culture

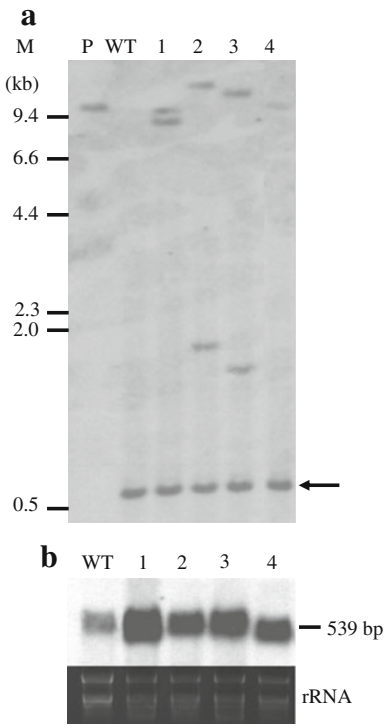

Fig. 5 Detection of a foreign gene *Hd3a* in transgenic lines by Southern blot analysis (**a**) and expression of *Hd3a* in transgenic lines by Northern blot analysis (**b**). *P*, constructed p1390UbiHd3a. *WT*, regenerated nontransformed plants. *1* and *2*, flowering cluster initiated directly from callus. *3* and *4*, leaves from different *Hd3a* transgenic flowering plants. *Arrow* indicates *Hd3a*-homologous sequence in *S. involucrata*

Comparing the band patterns between WT and *Hd3a* transgenic lines, we concluded that one T-DNA copy had integrated into transgenic line 4, while lines 1, 2, and 3 had two copies (Fig. 1b). These results also indicated that lines 1, 2, 3, and 4 derived from independent transgenic events.

Northern blot analysis of transgenic lines with *Hd3a* probe showed the presence of a single, expected 539-bp transcript in transformed lines (Fig. 5b). A hybridization signal also appeared in nontransformed plant, indicating that an ortholog of *Hd3a* is expressed in leaves of *S. involucrata*. Compared with the intensity of the hybridization signal of WT, the transgenic lines showed higher intensity of hybridization signals (Fig. 5b). These results obviously indicated that the early-flowering phenotype in the *Ubiquitin::Hd3a* transgenic *S. involucrata* was due to overexpression of the rice *Hd3a* gene.

Discussion

Fu et al. (2004, 2005b) first reported a hairy root system for *S. involucrata* using an *A. rhizogenes*-mediated transformation method, and successfully transferred chalcone isomerase (*CHI*) from *S. medusa* into the genome of *S. involucrata* to produce higher levels of flavonoids from hairy roots (Li et al. 2006). Intact plantlets could be regenerated from hairy roots; however, further molecular evidence confirming integration of target gene in the genome of the regenerated plants has not been described (Fu et al. 2004). Our transformation method is based on an *Agrobacterium*-mediated leaf disc transformation. Starting from the original seeds, it is possible to obtain transgenic plants growing on soil within 6 months. Since *S. involucrata* is a rare plant that

lives under specific environmental conditions, its seeds are naturally scarce. Leaves of the regenerated shoots could be also used as explants for genetic transformation using our transformation method, the transformation frequency being the same as when using leaf explants from seedlings. On the other hand, about 20% brown leaf explants caused by hygromycin selection were lost because of bacteria overgrowth. To improve the transformation efficiency, it is better to transfer hygromycin-resistant calli from the brown explants to fresh media as soon as possible.

As wild *S. involucrata* grows only in Alpine regions, near or above the snowline, cultivation of this species in a normal climate has not been reported so far. More recently, Zhao and Wang (2008) reported that regenerated plants could successfully survive and blossom in the third year after transplanting to Taishan Mountain; however, the cultivation conditions have not been described. We tried to plant the transgenic plantlets in the plant growth chamber at 17 ± 1°C; about 75% of the transgenic plants could survive under these conditions after 45 days of transplanting. Optimization of cultivation conditions for *S. involucrata* is now under further investigation.

S. involucrata is a valuable medicinal herb, well known for its flavonoids (Zhao et al. 2001; Fu et al. 2005a; Chu et al. 2006) and high tolerance to harsh, very cold Alpine climate with extremely high ultraviolet radiation and drought. The efficiency of this transformation protocol will allow us to easily generate mutants up- or downregulated for various key enzymes involved in flavonoid biosynthetic pathways (Cheng et al. 2007) and abiotic stress response pathways, and to understand the regulation or accumulation of specific flavonoid metabolites and its abiotic stress tolerance. A new set of mutants has recently been produced via dominant negative suppression of a rice *MutL* homolog, *OsPMS1*, using this transformation method.

Basic biological studies of *S. involucrata* are limited due to the difficulty of the reproductive success of this valuable plant under normal growth conditions. However, in flowering plants, the flower is the most important plant part for reproduction. An efficient flower regeneration protocol for *S. involucrata* is presented herein for the first time, with modified *Hd3a* expression under the control of the maize *Ubiquitin* promoter using our transformation method.

More recently, *Hd3a* and its orthologs have been proposed as florigens, or mobile flowering signals (Corbesier et al. 2007; Lin et al. 2007; Tamaki et al. 2007; Notaguchi et al. 2008; Xi and Yu 2009). Overexpression of *Hd3a* or its ortholog has been shown to cause an early-flowering phenotype in *Arabidopsis*, poplar (Böhlenius et al. 2006; Hsu et al. 2006; Zhang et al. 2010), rice (Tamaki et al. 2007), trifoliate orange (Endo et al. 2005), and European pear (Matsuda et al. 2009). Flower-like structures initiated directly from the stem explant transformed with *35S::PtFT1* within 4 weeks (Böhlenius et al. 2006), and flowers appeared early in transgenic plants (Kobayashi et al. 1999; Kojima et al. 2002; Endo et al. 2005; Böhlenius et al. 2006; Hsu et al. 2006; Tamaki et al. 2007; Matsuda et al. 2009). Our results also showed that overexpression of *Hd3a* accelerated flowering in *S. involucrata*. In addition to its early-flowering phenotype, the transgenic *Hd3a* callus could differentiate directly into flower cluster, and it took the callus less time to differentiate into flower than to differentiate into buds. However, these phenomena have not been described in plants overexpressing *FT* or its ortholog (Kobayashi et al. 1999; Kojima et al. 2002; Endo et al. 2005; Böhlenius et al. 2006; Hsu et al. 2006; Tamaki et al. 2007; Hou and Yang 2009; Matsuda et al. 2009).

The flower of *S. involucrata* is a precious source of medicines and plays an important role in reproduction. The efficiency of our flower regeneration system will allow us to easily generate flower in vitro for biological studies of flower development, bioactive phytochemicals in flower, and rapid mass production of flowers for medicinal purposes. Since the *Ubiquitin* promoter-driven *Hd3a* expression is so strong in *S. involucrata*, causing flowers to initiate directly from callus, we are now producing transgenic plants with modified *Hd3a* expression under an abiotic stress-inducible promoter, *rd29A*.

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