

Efficient transformation of *Kalanchoe blossfeldiana* and production of male-sterile plants by engineered anther ablation

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Abstract Engineered male sterility in ornamental plants has many applications such as facilitate hybrid seed production, eliminate pollen allergens, reduce the need for deadheading to extend the flowering period, redirect resources from seeds to vegetative growth, increase flower longevity and prevent gene flow between genetically modified and related native plants. We have developed a reliable and efficient *Agrobacterium*-mediated protocol for the genetic transformation of different *Kalanchoe blossfeldiana* commercial cultivars. Transformation efficiency for cv. ‘Hillary’ was 55.3% whereas that of cv. ‘Tenorio’ reached 75.8%. Selection was carried out with the *nptII* gene and increasing the kanamycin concentration from 25 to 100 mg l⁻¹ allowed to reduced escapes from 50 to 60% to virtually 0%. This method was used to produce male-sterile plants through engineered anther ablation. In our approach, we tested a male sterility chimaeric gene construct (*PsEND1::barnase*) to evaluate its effectiveness and effect on phenotype. No significant differences were found

in the growth patterns between the transgenic lines and the wild-type plants. No viable pollen grains were observed in the ablated anthers of any of the lines carrying the *PsEND1::barnase* construct, indicating that the male sterility was complete. In addition, seed set was completely abolished in all the transgenic plants obtained. Our engineered male-sterile approach could be used, alone or in combination with a female-sterility system, to reduce the invasive potential of new ornamentals, which has become an important environmental problem in many countries.

Keywords *Kalanchoe blossfeldiana* · Engineered male sterility · *PsEND1* promoter · Barnase · Anther ablation · Biosafe ornamentals

Abbreviations

AS	Acetosyringone
BA	6-Benzylaminopurine
CEF	Cefotaxime
<i>Gfp</i>	Green fluorescent protein
IAA	Indole-3-acetic acid
KAN	Kanamycin sulfate
KIN	Kinetin
LB	Luria Bertani medium
<i>Luc</i>	Luciferase gene
MES	2-(<i>N</i> -morpholino)ethane sulfonic acid
MS	Murashige and Skoog medium
NAA	α -Naphthalene acetic acid
<i>nptII</i>	Neomycin phosphotransferase gene
PCR	Polymerase chain reaction
TDZ	Thidiazuron
TIM	Timentin (ticarcillin/clavulanic acid)
<i>uidA</i>	β -Glucuronidase gene
SEM	Scanning electron microscopy

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Introduction

In the last years, new traits have been introduced into ornamental plants through conventional breeding. Currently, genetic engineering enables specific alterations of single traits in already successful varieties. Male sterility has many useful applications in horticultural crops. The availability of male-sterile plant varieties is relevant to produce hybrid plant lines which are more vigorous than the corresponding parental pure lines because of the phenomenon known as heterosis and to prevent gene flow between genetically modified and related native plants. In ornamental crops, the inability of a plant to set seed could reduce the need for deadheading to extend the flowering period, eliminate nuisance fruit, remove allergy producing pollen and increase flower longevity. Deadheading refers to the removal of dead or spent flowers either to encourage more flowering or to improve the general appearance of the plant. Most annuals and many perennials will continue to bloom throughout the growing season if deadheaded. Deadheading can be accomplished by pruning the spent flowers or even by pinching them off with the fingers. For plants with a profusion of tiny flowers, it is often easier to deadhead by shearing back the whole plant. It will take a little longer to set new flowers. Flowers that repeat bloom will often do so only if the old, dying flowers are removed. If they remain on the plant, they will go to seed and stop producing flowers. Even many flowers that bloom only once per season benefit from deadheading because the plant puts its energy into strengthening itself instead of producing seeds.

Kalanchoe blossfeldiana Poelln ($2n = 34$) belongs to the *Crassulaceae* family and it is native to Madagascar (Van Voorst and Arends 1982). The plant is a dark green, succulent perennial with scallop-edged leaves and large umbels of flower clusters held above the foliage. The upright, much-branched growth habit and tolerance of low moisture conditions makes it ideal for groundcover use, rock gardens, raised planters, or containers. The fleshy, dark shiny green leaves will reach 7.7 cm long by up to 3.8 cm wide with lobed edges. Many hybrid forms are available today coming in a variety of sizes and floral colors that reach from dwarf plants only 15 cm tall to plants over three times taller. Floral colors range from the traditional red to yellow, orange, salmon, to pink and almost any color in between. In the greenhouse, the plants bloom starting in December and last 6–8 weeks and they can easily be propagated from cuttings in the spring. It is a very popular pot plant because of its plentiful flowering and little need for care. *Kalanchoe* is the most grown flowering pot plant in Europe. In total there is a production of about 65 million plants yearly (Kalanchoe Growers Holland, <http://www.kalanchoe.nl>). There is a substantial

interest in the production of this ornamental plant as well as in the continuous development of cultivars with new traits making the plant more attractive for the consumer or contributing to reduce the production costs. Recently, several interesting traits have been introduced via genetic engineering in *K. blossfeldiana* leading to the production of dwarf genotypes, more compact growing plants, root inducing (Ri)-lines, plants with reduced ethylene sensitivity in flowers and marker-free transgenic plants (Christensen et al. 2008; Sanikhani et al. 2008; Topp et al. 2008; Thirukkumaran et al. 2009). However, there is not a reliable and efficient male-sterility system developed to this plant to prevent undesirable horizontal gene flow of the introduced transgenes and most of the transformation protocols reported previously for this species showed low efficiency, high escapes rate and transgene silencing (Aida and Shibata 1996, 1998).

Our engineered male-sterility approach was created using the *barnase* gene from *Bacillus amyloliquefaciens* (Hartley 1988) fused to the *PsEND1* promoter (Roque et al. 2007) to ablate specific tissues in the anther. Barnase has been used as an ablation agent in a variety of biotechnological applications, including the production of nuclear male sterility (De Block et al. 1997; Mariani et al. 1990; Zhan and Cheung 1996; Roque et al. 2007), nuclear female sterility (Goldman et al. 1994; Kandasamy et al. 1993) and a combination of both nuclear male and female sterility (Gardner et al. 2009). *Barstar* expression, an inhibitor of the ribonuclease barnase, has been used to restore fertility to plants with barnase-induced sterility (Mariani et al. 1992) and to prevent the effects of a possible ectopic barnase expression in engineered male and female-sterile plants (Gardner et al. 2009). Different approaches have created male-sterile lines using barnase expression under the control of a wide range of promoters. For example, the tapetal-specific promoter from the *TA29* gene of tobacco was used to target expression of barnase, resulting in the creation of male-sterile *Nicotiana tabacum* and *Brassica napus* plants (Mariani et al. 1990). The tobacco *TA56* promoter, which directs expression to the stomium and other areas of the anther involved in dehiscence, was used in conjunction with barnase by Beals and Goldberg (1997) to create male-sterile tobacco lines. Male sterility was induced in tobacco plants using a heterologous pollen-specific promoter from rice, *PSI*, to direct *barnase* expression (Zhan and Cheung 1996). Wheat lines that displayed male sterility were developed by transforming plants with barnase under control of tapetal-specific promoters from corn and rice (De Block et al. 1997).

The spatial and temporal *barnase* expression is very important for creating effective male sterility. In our study, *barnase* expression was controlled by the *PsEND1* promoter (Roque et al. 2007) with specific expression in those

tissues involved in the anther architecture of many plant species (Gómez et al. 2004; Roque et al. 2007; Pistón et al. 2008). *PsEND1* is a pea anther-specific gene that displays very early expression in the anther primordium cells. Later on, *PsEND1* expression becomes restricted to the epidermis, connective, endothecium and middle layer, but it is never observed in the anther filament, tapetal cells or microsporocytes. The expression pattern of this gene continues till floral anthesis. We fused the *PsEND1* promoter region to the cytotoxic *barnase* gene to induce specific ablation of the cell layers where the *PsEND1* is expressed and consequently to produce male-sterile plants. Expression of the chimaeric *PsEND1::barnase* gene in two Solanaceae (*Nicotiana tabacum* and *Solanum lycopersicum*) and two Brassicaceae (*Arabidopsis thaliana* and *Brassica napus*) species, impairs anther development from very early stages and produces complete male-sterile plants (Roque et al. 2007). The novelty of our approach resides in the use of the *PsEND1* promoter, instead of a tapetum-specific promoter, to produce the ablation of specific cell lines during the first steps of the anther development. The *PsEND1::barnase* chimaeric construct arrests the microsporogenesis before differentiation of the microspore mother cells; therefore no viable pollen grains are produced. This strategy represents an excellent alternative to generate genetically engineered male-sterile plants, having a potential interest to prevent undesirable horizontal gene flow in many plant species (Roque et al. 2007).

The main objectives of this study were to develop a reliable and efficient *Agrobacterium*-mediated protocol for the genetic transformation of *K. blossfeldiana* commercial cultivars and to engineer male sterility in this ornamental crop by ablating tissues essential for anther development and subsequently for pollen growth. A chimaeric construct that contained the cytotoxic RNase *barnase* gene under control of the anther-specific promoter *PsEND1* from pea (Roque et al. 2007), was introduced into two commercial cultivars of *K. blossfeldiana* to produce male-sterile plants. The efficacy of the sterility gene was corroborated by examining phenotypic changes in the transgenic anthers and testing the absence of viable pollen grains into the pollen sacs by scanning electron microscopy (SEM).

Materials and methods

Plant material and tissue culture

Two cultivars of *K. blossfeldiana*, obtained from local nurseries (Viveros Vangarden, Valencia), were used in these studies: ‘Hillary’ (white flower) and ‘Tenorio’ (red flower). Two sources of explants were used as starting materials. In the first, leaf and stem explants were prepared

from greenhouse grown mother plants. In the second, these organs were harvested from axenically grown plantlets. Young leaf explants (from the first to the fifth leaf from the apex) and shoot segments with axillary buds were collected from 3 to 4 months old greenhouse plants. Explants were first washed thoroughly with water, and then with 70% ethanol for 1 min. They were then surface sterilized by immersion in a 2.5% solution of sodium hypochlorite with 0.1% of 7X-O-matic detergent (Flow Laboratories) for 20 min and rinsed three times with sterile distilled water.

For tissue culture the basal medium (BM) consisted of Murashige and Skoog (MS) (Murashige and Skoog 1962) basal medium supplemented with 30 g l⁻¹ sucrose, 1 mg l⁻¹ thiamine-HCl, 100 mg l⁻¹ myo-inositol, Staba vitamins (Staba 1969) and 8 g l⁻¹ agar (Agar Bacteriológico Europeo, Pronadisa). The pH was adjusted to 5.7 before autoclaving. All antibiotics were filter sterilized and added to cooled media (45°C) before pouring into 9 cm diameter Petri dishes as 25 ml medium per plate. After surface sterilization, leaves from greenhouse plants were cut into 1–2 cm² pieces and cultured on regeneration medium (RM), consisting of BM supplemented with 1.0 mg l⁻¹ NAA and 2.0 mg l⁻¹ BA, with the abaxial surface in contact with the medium. In each assay, 35 explants were used as the starting material. Axenic plants were established by surface sterilization of 2 cm shoot segments processing an axillary bud excised from greenhouse grown plants. These nodal cuttings were cultured on propagation medium (PM) composed of BM supplemented with 0.1 mg l⁻¹ IAA in 15 × 2.5 cm culture tubes with 15 ml medium. Axenic plants obtained from shoots segments were propagated every month in 580 ml culture vessels with 40 ml PM and maintained as in vitro stocks plants. Leaves were excised from 2-month-old axenic plants, cut into 1–2 cm² pieces and cultured on the RM with the abaxial surface in contact with the medium. All cultures were incubated at 25°C under a 16 h photoperiod with fluorescent light (60 μmol m⁻² s⁻¹ intensity).

Agrobacterium tumefaciens strain and chimaeric gene constructs

Agrobacterium strain LBA4404 was used in all transformation experiments. LBA4404 was engineered to carry different plasmids: (i) a pBIN19 binary vector harboring, from the right to the left border, the *nptII* marker gene under the control of the *nos* promoter and the *nos* terminator, and the *gfp-S65T* (Chiu et al. 1996) reporter gene under the control of a 2X 35SCaMV constitutive promoter and the *nos* terminator; (ii) plasmid pCAMBIA2301 (http://www.cambia.org/daisy/bioforge_legacy/3726.html) harboring, from the right to the left border, the reporter gene *uidA-intron* under the control of the 35SCaMV

promoter and the *nos* terminator, and the *nptII* marker gene under the control of the 35SCaMV promoter and the 35SCaMV terminator; (iii) a plasmid derived from pBI101 harboring, from the right to the left border, the *nptII* marker gene under the control of the *nos* promoter and the *nos* terminator, and the *barnase* gene under the control of the *PsEND1* promoter and the *nos* terminator. Bacteria were grown at 28°C on solid LB plates supplemented with 40 mg l⁻¹ rifampicin and 100 mg l⁻¹ kanamycin. A single colony was used to inoculate 25 ml of LB liquid medium with the same antibiotics. Flasks were maintained at 28°C and 200 rpm for 24 h and later on were used to inoculate a liquid MS medium supplemented with 20 g l⁻¹ sucrose, 100 mg l⁻¹ myo-inositol, 1 mg l⁻¹ thiamine-HCl, 500 mg l⁻¹ 2-(*N*-morpholino)ethane sulfonic acid (MES) and 0.2 mM acetosyringone (AS) dissolved in 70% ethanol (sterilized by filtration), which was cultured at 28°C for 12 h. Inoculation of explants was conducted when the bacterial culture reached an OD (600 nm) of 0.06.

The promoter region of the *PsEND1* gene (GenBank accession no.: AY324651) was previously cloned into the binary vector pBI101 where the -2736/-6 promoter fragment was fused to the coding sequence of the β -glucuronidase (*uidA*, *GUS*) reporter gene (Gómez et al. 2004). *PsEND1::barnase* chimaeric gene: primers Ribo1 (5'-TAGG ATCCCCGACCATGGCACAGGTTATC-3') and Inhi2 (5'-G CGAGCTCTTAAGAAAGTTGATGGTGATG-3') were designed based on the published sequence of *barnase* and *barstar* genes (Hartley 1988) to amplify the *barnase-barstar* fragment previously cloned into the *Bam*HI site of pBlue-script KS (+). *Barnase* is a very active ribonuclease. Even a low level of expression from aberrant promoter sequences or run-off expression from neighboring genes during manipulations in *E. coli* or *Agrobacterium* would have prevented the survival of the bacteria. Therefore, the *barstar* gene which encodes an inhibitor of *barnase* is included in the construct. The Ribo1 and Inhi 2 primers introduce *Bam*HI and *Sac*I restriction sites, respectively. The polymerase chain reaction (PCR) resulting fragment was cloned into the pGEM-T Easy (Promega) and later released with the *Bam*HI and *Sac*I enzymes. The *Bam*HI-*Sac*I fragment was cloned by replacement of the *uidA* coding sequence into the binary vector pBI101 generating the pBI101-*PsEND1::barnase-barstar* construct (Fig. 3a). The *nos::nptII* plant selectable marker gene, which confers resistance to kanamycin in transgenic plants, was also introduced in the T-DNA.

Transformation and regeneration of transgenic plants

Transformation experiments were carried out with *K. blossfeldiana* cv. 'Tenorio' and cv. 'Hillary'. Leaf explants were prepared from 2-month-old axenic plants as described above. Explants were inoculated in groups of 25

explants with 25 ml of the bacterial suspension for 5 min and thereafter transferred to co-culture medium consisting of RM supplemented with 0.2 mM AS. Explants were co-cultured for 7 days in the dark at 25°C, afterwards they were transferred into sterile glass jars containing a liquid washing medium consisting of BM containing 600 mg l⁻¹ cefotaxime and soaked for 5 min. The explants were subsequently blotted dry with sterile filter paper and subcultured onto RM supplemented with 300 mg l⁻¹ timentin and 25 mg l⁻¹ kanamycin for *Agrobacterium* eradication and selection of transgenic events, respectively. Control explants were treated in the same manner, except for the inoculation with *Agrobacterium*. Control groups were established and cultured on medium with and without kanamycin. All explants were subcultured every 2 weeks onto the same fresh medium until shoots were long enough to be separated from the callus. Individual putative transgenic regenerants were transferred to PM supplemented with 50 mg l⁻¹ kanamycin in order to test their ability to form roots under selective conditions. In each assay, 42 explants were used as the starting material.

The transformation efficiency was estimated as the number of independent transformation events (one transgenic plant per explant) in relation to the total number of inoculated explants. Regenerated plants with well-developed roots were transferred to plastic pots or plug trays containing peat moss as substrate. Plantlets were cultivated in growth chambers and initially covered with a transparent plastic to maintain high humidity. Plants were maintained under long day conditions (16 h light/8 h dark photoperiod) for 4 months and then transferred to a greenhouse with short day conditions (10 h light/14 h dark photoperiod) where they flowered 1 month later.

PCR analysis

Genomic DNA was extracted from leaves according to Rogers and Bendich (1994). PCR detection of the *nptII*, *uidA* and *gfp* transgenes was performed with standard methodologies as follows. For each sample, 1 μ l (0.1–0.2 μ g) DNA was incubated in a final volume of 20 μ l containing 0.2 mM of each dNTPs, 1.5 mM MgCl₂, 0.6 μ M 5' and 3' primers and 0.5 U Taq DNA polymerase. The conditions for amplification were as follows: for *nptII*, 30 cycles of 30 s at 94°C for denaturation, 45 s at 56°C for annealing and 1 min at 72°C for extension; for *uidA*, 30 cycles of 30 s at 94°C for denaturation, 45 s at 60°C for annealing and 1 min 10 s at 72°C for extension; for *gfp*, 35 cycles of 30 s at 94°C for denaturation, 30 s at 60°C for annealing and 1 min at 72°C for extension. Primer sequences were: for *nptII*, KAN-1: 5'-AAG ATG GAT TGC ACG CAG GTT C-3' and KAN-2: 5'-GAA GAA CTC GTC AAG AAG GCG A-3'; for *uidA*, GUS-1: 5'-ATC

AGG AAG TGA TGG AGC ATC A-3' and GUS-2: 5'-GGT GAT CGG ACG CGT CGG GTC G-3'; for *gfp*, GFP-D: 5'-ATG GTG AGC CAA GGG CGA GGA-3' and GFP-R: 5'-GGA CCA TGT GAT CGC GCT TC-3'. PCR products were detected by UV light after electrophoresis on 1% w/v agarose ethidium bromide gels. Predicted products sizes were 781 bp for *nptII*, 1021 bp for *uidA* and 661 bp for *gfp*.

The presence of the *barnase* transgene (544 bp) was detected by PCR using the primers: Ribo3 (5'-ACG GAC CAT TAT CAG ACC TTT AC-3' and Inhi3: (5'-CGC AGC CTT CCG CTT TCG C-3'. DNA sequences were analyzed in an ABI PRISM 377 (Perkin Elmer) automatic sequencer.

Southern analysis

Genomic DNA was extracted from leaves according to Dellaporta et al. (1983). 40 µg of DNA was digested with *EcoRI* and separated by electrophoresis in a 1% w/v agarose gel. DNA was transferred to a nylon membrane (Nytran, Schleicher & Schuell), fixed by UV irradiation and hybridized with a digoxigenin-labeled 661 bp probe of the coding region of the *gfp* gene, prepared by PCR according to the supplier instructions (Roche). Detection of hybridised DNA fragments was conducted by incubation of the membranes with alkaline phosphate conjugated anti-digoxigenin followed by chemiluminescent substrate CSDP and exposition to an X-ray film. All steps were performed following the supplier instructions (Roche).

Ploidy level analysis

The ploidy level was evaluated by flow cytometry (Smulders et al. 1995). Leaf tissue from in vitro plants was used for nuclei isolation. Pieces of tissue (1 cm²) were chopped individually on a glass plate with a sharp razor blade in 200 µl of nuclei isolation buffer (Partec). The sample was then passed through a 50 µm nylon filter and 800 µl of staining solution (Partec), containing 1 mg l⁻¹ DAPI (4,6-diamino-2-phenyl-indole), were added for DNA fluorescence. The DNA content of the isolated nuclei was measured using a Partec PAS-II flow cytometer equipped with a mercury lamp. Fluorophore excitation peak is below 420 nm and fluorescence emission peak for DAPI is between 435 and 500 nm. The data were plotted on a histogram where the horizontal axis shows DNA content (proportional to fluorescence) and the vertical axis shows nuclei number. About 5,000–10,000 nuclei were measured per sample. Analyses were carried with cv. 'Tenorio': 72 in vitro stocks plants, 154 regenerated plants and 95 transgenic plants.

Light microscopy and scanning electron microscopy

Transformed explants were examined periodically for *gfp* expression under a fluorescence stereomicroscope (Leica MZ FLIII) equipped with a Leica Fluorescence Module GFP3 comprising a 470–440 nm Excitation Filter and a 525–550 nm Barrier Filter. A mercury lamp provided the light source. The red autofluorescence from chlorophyll was not blocked with any filter. Floral buds and stamens from both transgenic and WT plants were freshly harvested and dissected using forceps and scalpel. Light photographs of dissected flowers and stamens were obtained using a stereomicroscope (MZ8, Leica).

β-Glucuronidase activity was determined histochemically according to Jefferson et al. (1987). Root, shoot and leaf segments from the putative transgenic plants were stained for 24 h at 37°C, cleared with 70% ethanol and observed under a stereomicroscope MZ8 Leica.

For SEM, fresh floral buds were vacuum infiltrated with a FAE solution (ethanol : acetic acid : formaldehyde : water; 50:10:3.5:26.5; v/v/v/v) for 15 min and fixed with fresh solution during 4–16 h at 4°C. The samples were dehydrated in an ethanol series and critical point dried in liquid CO₂ (Polaron E300). The specimens were mounted on stubs and several outer whorl organs (sepals and petals) of individual flowers were removed manually. The samples were then coated with gold–palladium (4:1) particles (200 nm) in a Sputter Coater SC500 (Baltec). SEM was performed with a Jeol JSM-5410 microscope (10 kV) and the images were processed using the programme Autobeam (ISIS, Oxford Instruments).

Pollen viability and germination tests

Pollen viability tests were performed in squeezed anthers using a solution of carmine acetate (0.5 g) in 45% acetic acid and diluted 1:1 with 30% glycerol. Viable pollen grains (stained in red) were observed and counted with an Eclipse E600 (Nikon) microscope. Pollen germination tests were performed in a medium composed of 0.292 M sucrose, 1.27 mM Ca (NO₃)₂, 1.62 mM H₃BO₃, 1 mM KNO₃, 0.1 mM KH₂PO₄ and 0.5% agarose. The pollen grains were incubated on glass slides coated with the solid medium in the dark during 2 h at 25°C. The germinated pollen grains were observed and counted using a Diaphot (Nikon) inverted microscope.

Morphological measurements

Morphological measurements of vegetative growth were made to determine whether the *barnase* expression in anthers would affect other growth parameters. Measurements were taken in the greenhouse on 11 T1 hemizygous

Table 1 Regenerating leaf explants (%) in *K. blossfeldiana*

Explant source	Cultivar	
	'Hillary'	'Tenorio'
Greenhouse plants	44.0 ± 10.1	74.3 ± 7.5
Axenic plants	100.0 ± 0.0	100.00 ± 0.0

Percentage of explants with adventitious shoots after 1 month culture in the regeneration medium (RM: BM with 1.0 mg l⁻¹ NAA and 2.0 mg l⁻¹ BA). In each assay, 35 explants were used as the starting material. Values represent mean ± SE

transgenic plants and 6 WT control plants. Plant height in flowering plants (distance from soil line to top of the tallest growing point), leaf length and width (average measurements from five fully expanded leaves), internodal length and number of inflorescences per plant were evaluated. Morphological measurements were taken over the course of several days on each plant as its first five flowers reached anthesis.

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) to evaluate significant differences between means. Means differing significantly were compared using Duncan's multiple range test at a 5% probability level. Data variability was expressed as the mean ± SE.

Results

Plant regeneration from leaf explants and selection with kanamycin

Leaf explants from greenhouse plants were surface-sterilized and cultured in different media supplemented with an

auxin (NAA or IAA used separately) and a cytokinin (BA) (data not shown). After 1 month, the best percentage of explants with adventitious shoots was obtained using BM containing 1.0 mg l⁻¹ NAA and 2.0 mg l⁻¹ BA was 44.0 for cv. 'Hillary' and 74.3 for cv. 'Tenorio' (Table 1). This medium was chosen as regeneration medium (RM). Axenic plants were obtained from greenhouse plants and propagated every month to the propagation medium (PM). Leaf explants were taken from these axenic plants and cultured in RM. By using axenic plants as the source for explants, the percentage of regenerating explants reached 100% in both cultivars (Table 1). In summary, cultivars tested gave a very good response with NAA and BA for adventitious shoot formation and explants derived from axenic plants gave a superior regenerating rate compared to explants derived from greenhouse plants.

Since the plasmids used for transformation experiments carried the *nptII* gene as selectable marker, it was necessary to determine the concentration of kanamycin for the suitable selection of transgenic events. Leaf explants from axenic plants were cultured in RM with different concentrations of kanamycin (0, 25, 50 and 100 mg l⁻¹). A concentration of 25 mg l⁻¹ was sufficient in order to inhibit the growth of non-transformed cells in leaf explants (Table 2) in both cultivars. In addition, leaf explants from axenic plants were cultured in RM and regenerated shoots were cultured in PM with different kanamycin concentrations (0, 25, 50 and 100 mg l⁻¹). According to our results (Table 2), a kanamycin concentration of 50 mg l⁻¹ was used in PM to select transgenic plants for both cultivars.

Genetic transformation experiments

Genetic transformation of *K. blossfeldiana* was conducted with axenic plants as a source of leaf explants as described previously. For the experiments 1 and 2, explants from cv. 'Tenorio' were inoculated with *Agrobacterium* strain

Table 2 Effect of kanamycin concentration on shoot regeneration from axenic leaf explants and rooting of regenerated shoots in *K. blossfeldiana*

Kanamycin concentration (mg l ⁻¹)	Regenerating leaf explants (%) ^a		Rooted shoots (%) ^b	
	'Hillary'	'Tenorio'	'Hillary'	'Tenorio'
0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0a	100.0 ± 0.0a
25	0.0 ± 0.0	0.0 ± 0.0	16.0 ± 3.7b	20.0 ± 4.1b
50	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0c	0.0 ± 0.0c
100	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0c	0.0 ± 0.0c

Different letters within a column indicate significant differences ($P = 0.05$) by Duncan's multiple range test

^a Percentage of explants with adventitious shoots after 1 month of culture in the regeneration medium (RM: BM with 1.0 mg l⁻¹ NAA and 2.0 mg l⁻¹ BA). In each assay, 42 explants were used as the starting material. Values represent mean ± SE

^b Percentage of regenerated shoots with roots after 1 month of culture in the propagation medium (PM: BM with 0.1 mg l⁻¹ IAA). In each assay, 42 explants were used as the starting material. Shoots were regenerated from axenic plants leaf explants in RM. Values represent mean ± SE

LBA4404 and cultured with 25 mg l^{-1} kanamycin. After 1 month of culture, explants began to develop adventitious shoots in selective medium, which were excised and cultured in PM. In some cases, several independent transformation events were identified within the same explant (adventitious shoots on opposite sides of the same explant) but only one plant per explant was recovered. Transgenic plants were able to root with 50 mg l^{-1} kanamycin. Transgenic plants were acclimatized in a growth chamber and transferred later to a greenhouse, where they subsequently flowered normally under short day conditions (Fig. 1a–g). In the experiment 1, 34 transgenic plants with the *uidA* gene (confirmed by PCR analysis) were obtained and the transformation efficiency was 11.2% (Table 3). However, a high percentage of explants with escapes (shoots regenerated with kanamycin that did not carry the *nptII* gene) appeared (65 plants out of 99 regenerated plants, which represents a 65.6% rate). Transgenic plants were analyzed for *uidA* expression (histochemical assay with X-Gluc) in different tissues (root, shoot and leaf segments). 32 out of 34 plants revealed *uidA* expression in all tested tissues and two plants had no *uidA* expression.

PCR analysis was performed in transgenic plants to detect the *nptII* and *uidA* genes. The presence of both genes was identified among all the plants tested with the exception of the two plants without *uidA* expression, in which only the *nptII* gene was detected. In this case, some partial transference of T-DNA may have occurred and only plants with a complete copy of the *nptII* were recovered with the kanamycin selection. In the experiment 2, 61 transgenic plants with the *gfp* gene were obtained and the transformation efficiency was 22.0% (Table 3). As in the case of the experiment 1, the escapes rate was high (53.4%). PCR analysis was performed and it revealed the presence of both genes (*nptII* and *gfp*) in all the plants (Fig. 1h, i). Southern analysis for the *gfp* plants confirmed the integration of the transgene and the presence in the transgenic plant of 1, 2 or 3 copies of the *gfp* gene (Fig. 1j). In the experiments 3 and 4, two cultivars of *K. blossfeldiana* ('Hillary' and 'Tenorio') were transformed with the *nptII* and *PsEND1::barnase* genes. The protocol was the same as in the experiments 1 and 2, except for the kanamycin dose for selection: the concentration of kanamycin was raised to 100 mg l^{-1} in RM. The developed shoots were transferred to PM with 50 mg l^{-1} kanamycin and transgenic plants were selected. In both cultivars, escapes were virtually zero (1 out of 92 for 'Tenorio' and 0 out of 93 for 'Hillary') and transformation efficiency was higher than in previous experiments, scoring 55.3% for 'Hillary' and 75.8% for 'Tenorio' (Table 3). Transgenic plants of *K. blossfeldiana* were produced with significantly high transformation efficiencies when compared to other transformation methods previously reported.

Ploidy level analysis

The ploidy level of the transgenic plants of *K. blossfeldiana* cv. 'Tenorio' was analyzed and the data were compared with those of the original materials. *K. blossfeldiana* originally has $2n = 2x = 34$ chromosomes. Modern cultivars have been obtained by inter-specific hybridization and usually they show high ploidy levels when compared with wild species. Neither the DNA content of the cultivars used here, nor a reference cultivar of known ploidy level was available to compare the ploidy pattern of each transgenic plant with the original one. Therefore, the $2C$ value was arbitrarily assigned to the first peak and next peaks were assigned with a relative value in relation to the first one. Flow cytometric analysis of leaf tissue revealed the presence of two ploidy patterns (Fig. 1k, l): the first one showed two G_0/G_1 phase-peaks at $2C$ and $4C$ ($2x$ plants) and the second showed two G_0/G_1 phase-peaks at $4C$ and $8C$ ($4x$ plants). All the plants from the original cultivar (in vitro stock plants) showed a $2x$ ploidy pattern. Transgenic plants showed both patterns (64% showed a $2x$ ploidy pattern and 36% showed a $4x$ ploidy pattern). Regenerated plants from control explants were found to have both patterns (94% showed a $2x$ ploidy pattern and 6% showed a $4x$ ploidy pattern). When plants were acclimatized, $2x$ plants were evidently distinguishable from $4x$ plants, the $2x$ had identical morphology to the original plants, while the $4x$ showed small growth rates and thicker dark green leaves (Fig. 1m, n). These results suggested that leaf tissue of cultivars used here has a polysomatic structure and regeneration from it leads to plants with increased ploidy levels. This does not exclude the possibility that duplication occurred during in vitro culture and/or transformation phase.

GFP as in vivo selectable marker

While the *nptII* gene was employed for selection of transgenic plants, the *gfp* gene expression was also examined during the transformation process in order to evaluate the ability of *gfp* as a selectable marker. The expression of the *gfp* gene in the leaf explants can be observed 3 weeks after inoculation (when adventitious buds begin to appear) and also in the transgenic plants obtained. However, some explants that initially showed fluorescence, did not regenerate any transgenic plants, and there were explants without initial fluorescence that regenerated transgenic plants (Table 4). The *gfp* expression in transformed cells should be useful to select transformation events at early stages, so that selectable marker genes may not be required. In the case of *K. blossfeldiana*, if the selection had been made based only on *gfp* expression, a significant number of transgenic plants could be undetected (Table 4). Probably,

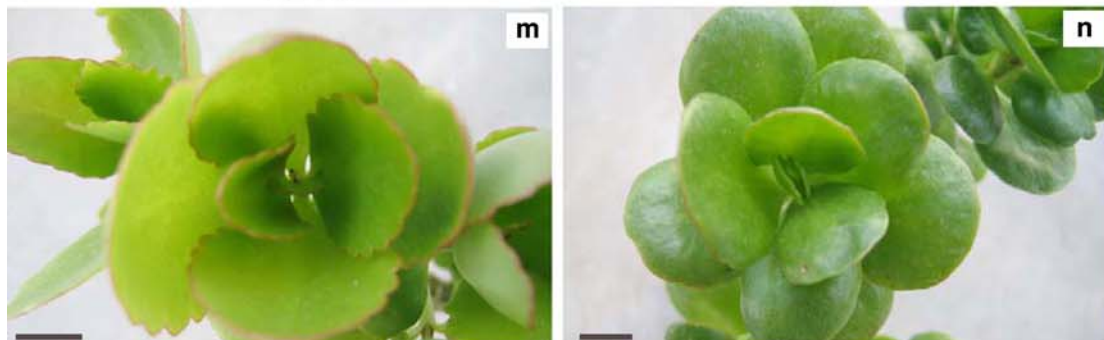
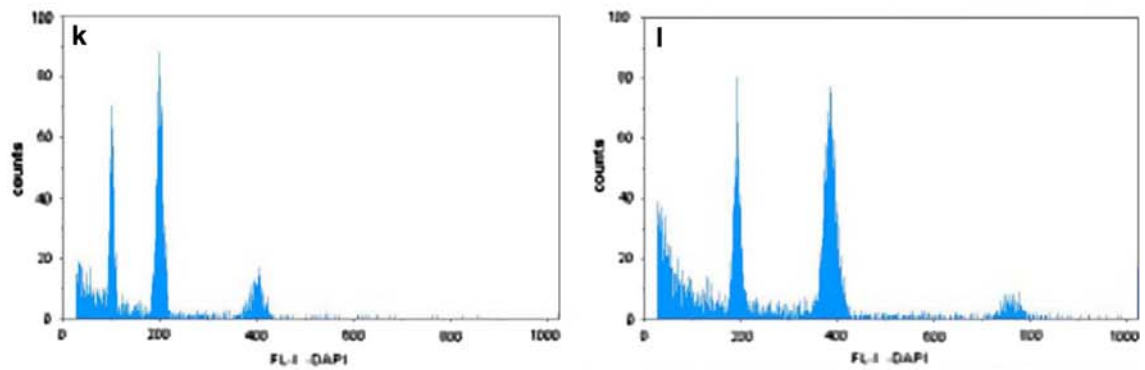
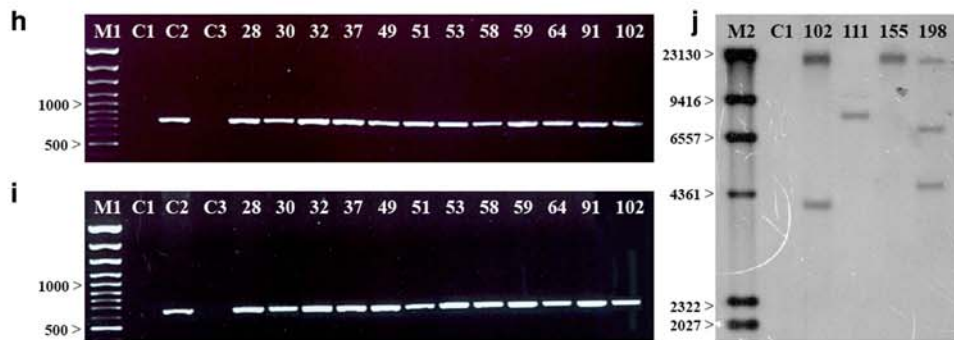
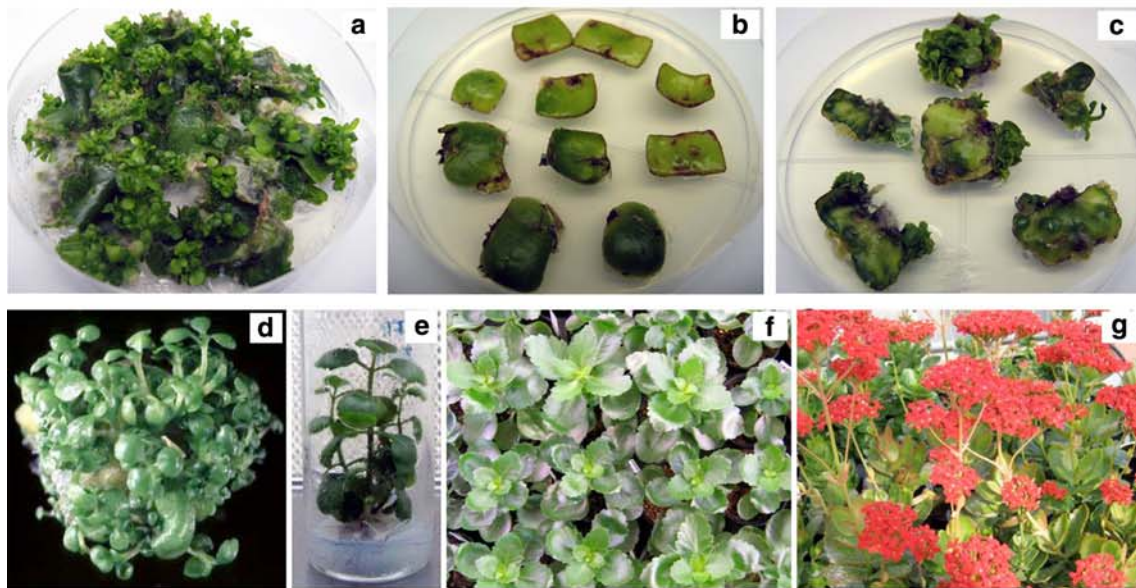


Fig. 1 Production of transgenic plants of *Kalanchoe blossfeldiana*. **a** Control explants cultivated in the regeneration medium without kanamycin (30 days). **b** Control explants cultivated in the regeneration medium with 100 mg l⁻¹ kanamycin (30 days). **c** *Agrobacterium* inoculated explants cultured with 100 mg l⁻¹ kanamycin (30 days). **d** In vitro regenerated transgenic shoots (45 days). **e** In vitro rooted transgenic plant (60 days). **f** Plant acclimatization in growth chamber (12 weeks). **g** Plants growing in the greenhouse (short day). Molecular analyses of *K. blossfeldiana* transgenic plants carrying *nptII* and *gfp* and genes. **h** PCR analysis for *nptII* gene with a fragment size of 781 bp. **i** PCR analysis for *gfp* gene with a fragment size of 661 bp. **j** Southern blot analysis of *EcoRI* digested genomic DNA (40 µg) hybridized with a *gfp* digoxigenin-labeled probe. *C1* negative control, DNA from a non transformed plant; *C2* positive control, plasmid DNA with both genes; *C3* reaction control, without DNA; *M1* DNA molecular weight marker, Gene Ruler 100 bp DNA Ladder Plus (Fermentas); *M2* DNA molecular weight marker II, digoxigenin-labeled, Roche. **k** Analysis of ploidy level in transgenic plants of *K. blossfeldiana* cv. ‘Tenorio’: ploidy pattern found in 2x plants. **l** Ploidy pattern found in 4x plants. **m** Phenotype of 2x plants. **n** Phenotype of 4x plants. Bars in **m**, **n** represent 1 cm

this problem is related to the fluorescence visualization in tissues with high chlorophyll content, like is the case of leaves. Chlorophyll shows strong red autofluorescence that could mask the green fluorescence of a few cells. In *K. blossfeldiana*, green fluorescence is clearly visible in the initial callus with a disorganized growth (whitish callus), while it becomes increasingly difficult to identify in the subsequent organogenic callus and in the adventitious buds (Fig. 2a–d). Green fluorescence was also observed in transgenic plants: for in vitro plants, tissues from root, shoot, and leaf were examined; for in vivo plants, flowers were also observed. All kanamycin resistant plants showed green fluorescence in different organs and tissues. Green fluorescence in transgenic plants was high in roots (Fig. 2e, f), in aerial roots from shoots (Fig. 2k, l) and in the petal abaxial surface (Fig. 2q, r). Green fluorescence was also visible in shoot sections (Fig. 2g, h), in leaves (Fig. 2i, j) and in flowers (Fig. 2m, n). Green fluorescence was not visible in the petal adaxial surface (Fig. 2o, p). Within the same organ, *gfp* expression varied in different tissues or cell types (e.g., shoot section, Fig. 2h). From these observations, we determine that there is not a complete

Table 4 Relationship between *gfp* expression in explants 3 weeks after transformation and *gfp* expression in transgenic plants

No. of <i>gfp</i> in explants ^a	No. of <i>gfp</i> in transgenic plants ^b	
<i>gfp</i> +		
129	No. of explants yielding <i>gfp</i> + plants	36 (28%)
	No. of explants yielding <i>gfp</i> - plants	35 (27%)
	No. of explants without plants	58 (45%)
<i>gfp</i> -		
148	No. of explants yielding <i>gfp</i> + plants	25 (17%)
	No. of explants yielding <i>gfp</i> - plants	35 (24%)
	No. of explants without plants	88 (59%)

^a Number of explants with *gfp* expression was recorded 3 weeks after transformation

^b When transgenic plants were obtained, it was recorded if the explant that had originated each transgenic plant had showed *gfp* expression 3 weeks after transformation

constitutive expression of the *gfp* gene, although it is certainly under the control of a strong constitutive promoter.

Selection of diploid male-sterile plants and morphological measurements

In experiments 3 and 4, we regenerated transgenic plants of two *K. blossfeldiana* cultivars carrying the construct pBI101-*PsEND1::barnase*. We used 168 leaf explants from the ‘Hillary’ cultivar and 120 leaf explants from the ‘Tenorio’ cultivar and obtained 93 and 91 transgenic plants for ‘Hillary’ and ‘Tenorio’ cultivars, respectively. We selected only the diploid plants and finally we retained 20 lines of each cultivar for further phenotypic and molecular analyses.

Morphological measurements of vegetative growth were taken over the course of several days on each plant as its first five flowers reached anthesis to determine whether the

Table 3 Results of transformation experiments in *K. blossfeldiana*

Exp	Cultivar	T-DNA genes	Kanamycin conc. (mg l ⁻¹)	No. of explants	No. of regenerated plants	No. of PCR positive plants	Transformation efficiency (%) ^a	Escapes rate (%) ^b
1	‘Tenorio’	<i>nptII</i> , <i>uidA</i>	25	304	99	34	11.2	65.6
2	‘Tenorio’	<i>nptII</i> , <i>gfp</i>	25	277	131	61	22.0	53.4
3	‘Hillary’	<i>nptII</i> , <i>barnase</i>	100	168	93	93	55.3	0.0
4	‘Tenorio’	<i>nptII</i> , <i>barnase</i>	100	120	92	91	75.8	1.1

^a The number of independent PCR positive transgenic plants (coming from different explants) divided by the number of inoculated explants

^b The number of non transgenic plants divided by the total number of regenerated plants from inoculated explants

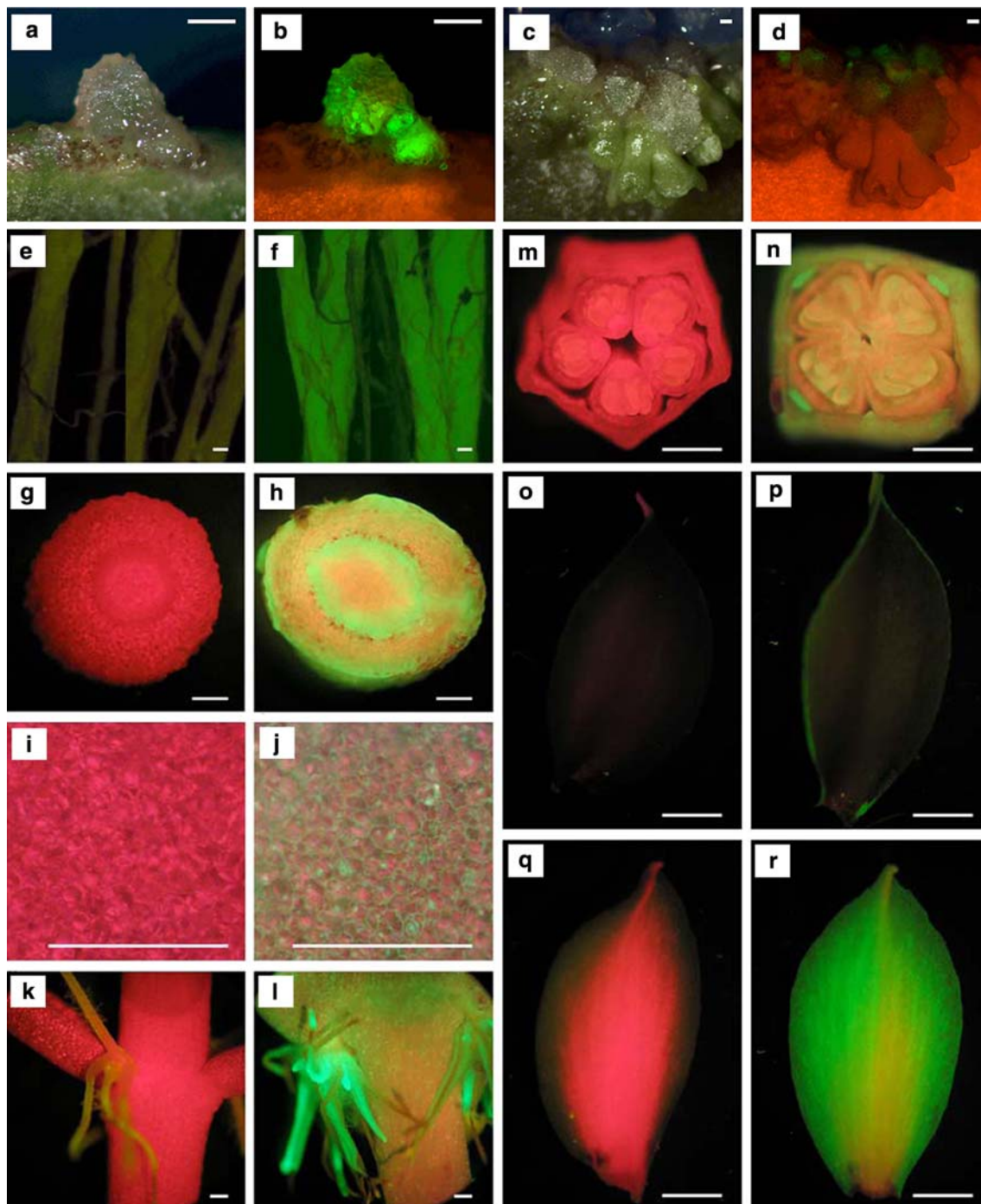


Fig. 2 Expression of *gfp* in explants of *K. blossfeldiana* during regeneration of transgenic plants. Initial callus with disorganized growth under either white light (a) or blue light (b). Subsequent organogenic callus with adventitious shoots under either white light (c) or blue light (d), e roots from control plant grown in vitro. f roots from transgenic plants, g shoot section from control, h shoot section from transgenic plants, i leaf segment from control, j leaf segment

from transgenic plants, k shoots with aerial roots from control, l shoots with aerial roots from transgenic plants. Plants grown in the greenhouse. m Flower section from control, n flower section from transgenic plants, o petal adaxial surface from control, p petal adaxial surface from transgenic plants, q petal abaxial surface from control, r petal abaxial surface from transgenic plants. Bar 1 mm

barnase expression in the anther tissues would affect other growth parameters. The measured parameters included plant height at flowering, leaf width, leaf length, node

number and number of inflorescences per plant. Our results indicated that the vegetative growth and flowering of the transgenic plants were not modified in a significant manner

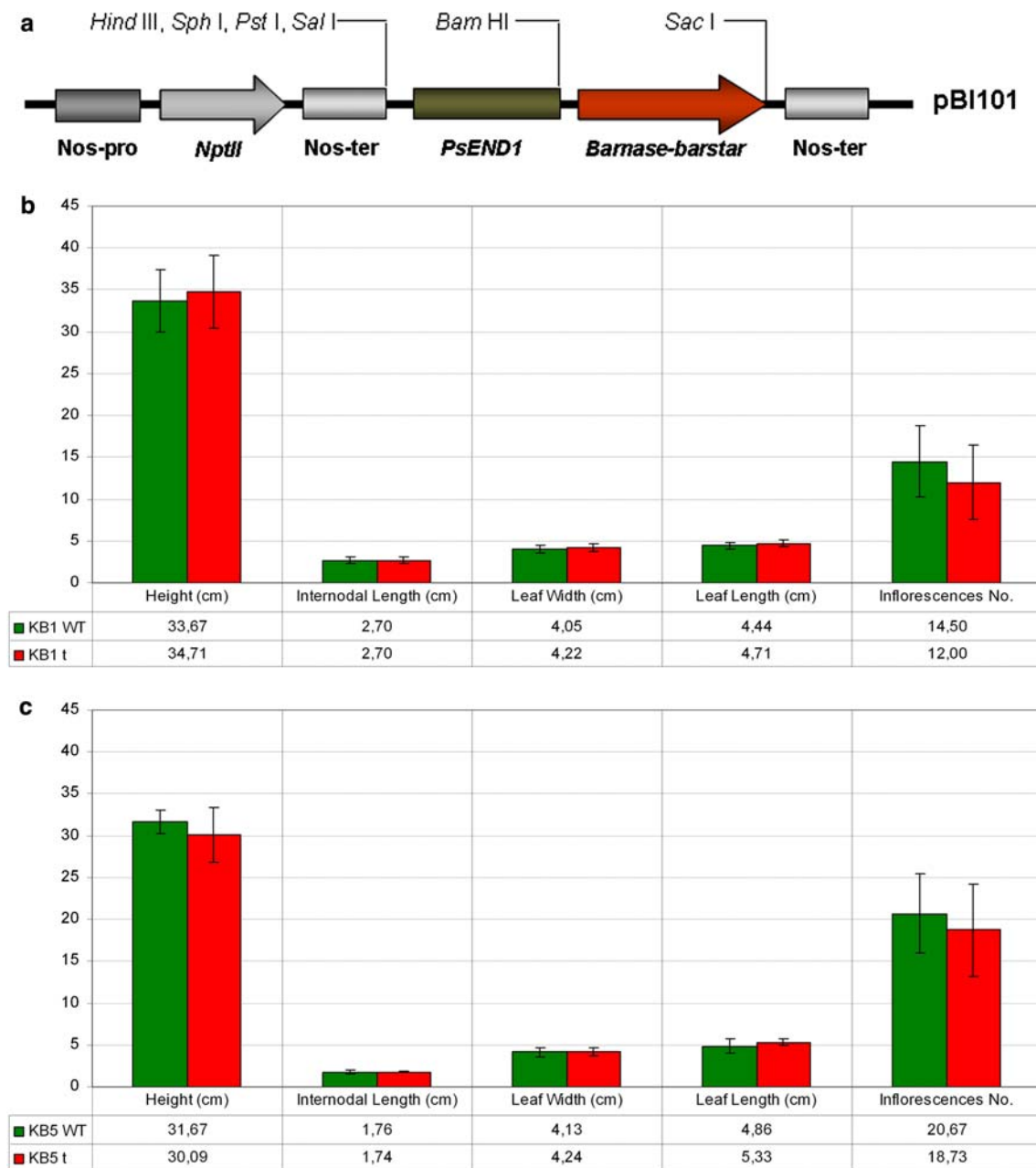


Fig. 3 a *PsEND1::barnase-barstar* chimaeric gene construct. **b–c** Morphological measurements of ‘Hillary’ (KB1) and ‘Tenorio’ (KB5) male-sterile plants versus WT control plants. The vegetative growth

and flowering of the transgenic plants were not modified in a significant manner by the barnase expression in the anthers LSD test $P < 0.05$

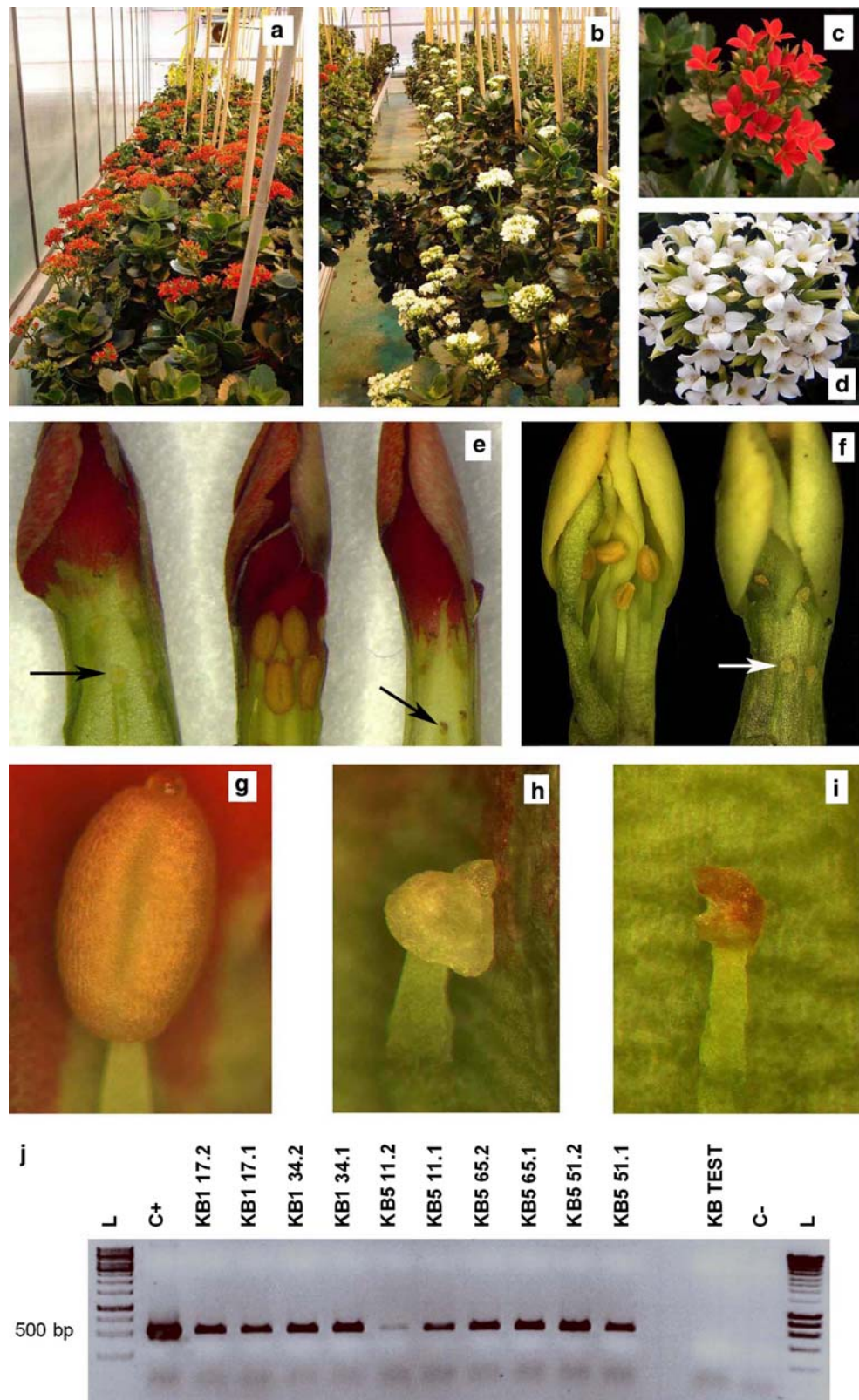
by the barnase expression in the anthers (Fig. 3b, c; LSD test $P < 0.05$). Therefore, it seems that there is no ectopic expression of the *barnase* gene in vegetative or reproductive plant tissues other than anthers, corroborating the tissue-specificity of the *PsEND1* promoter.

Early anther ablation results in efficient male sterility

Transgenic ‘Tenorio’ (Fig. 4a, c) and ‘Hillary’ (Fig. 4b, d) plants showed normal vegetative development and

flowering. However, anthers from transgenic lines carrying the chimaeric gene construct *PsEND1::barnase* compared with non transformed anthers from WT plants showed dramatic differences in development. Anthers at different stages of development were examined by light and SEM. In WT anthers from flowers 1 day prior to anthesis (Fig. 4e center and Fig. 4f left), the locules were fully developed, showing the normal shape (Fig. 4g) and containing viable pollen grains, whereas the transgenic ones showed small structures at the end of a short filament (stick-like) in the

Fig. 4 Phenotypes of the male-sterile *Kalanchoe blossfeldiana* plants transformed with the construct pBI101-*PsEND1::barnase*. **a** Male-sterile lines of the ‘Tenorio’ cultivar in the greenhouse. **b** Male-sterile lines of the ‘Hillary’ cultivar in the greenhouse. **c** Inflorescence of a ‘Tenorio’ male-sterile line showing apparently normal red flowers. **d** Inflorescence of a ‘Hillary’ male-sterile line showing apparently normal white flowers. **e** Flowers from a ‘Tenorio’ WT plant (*center*) and two male-sterile lines (*left*, *right*) 1 day prior to anthesis. In the WT flower the locules and pollen grains were fully developed, showing the normal shape, whereas the transgenic ones showed small structures at the end of the filament (*stick-like*) in place of a four-lobed anther (*black arrows*). **f** Flowers from a ‘Hillary’ WT plant (*left*) and a male-sterile line (*right*) 1 day prior to anthesis with ablated anthers (*white arrow*). **g** WT anther from a ‘Tenorio’ plant showing the normal four-lobed shape. **h** Close-up of an ablated anther. The locules of sterile anthers were narrow and unexpanded (*stick-like* structures). **i** In most cases the undeveloped anther tissues were necrotic, which represented the ablated anther tissues and aborted pollen grains. The anther filaments of the transgenic plants were short when compared with WT filaments. **j** PCR analysis of ten selected male-sterile lines by amplification of the *barnase* gene with primers *RiboIII* and *InhIII* (544 bp). C+ positive control, *PsEND1::barnase* construct; C- negative control, water; L molecular weight markers; KB1 cv. ‘Hillary’; KB5 cv. ‘Tenorio’; KB TEST non-transformed plant



place of a four-lobed anther (Fig. 4e left and right, black arrows; Fig. 4f right, white arrow) with a fully expanded filament.

No pollen grains were observed in the modified anther structures developed instead of normal anthers in the lines carrying the male-sterility construct. The locules of sterile

anthers from flowers 3–4 days prior to anthesis were narrow and unexpanded (Fig. 4h). In most cases the undeveloped anther tissues become a necrotic structure composed of the ablated anther tissues and aborted pollen grains (Fig. 4i). The cross-pollination of the male-sterile plants with pollen from wild-type plants produced normal fruits and seeds. These results indicate that female fertility was unaffected in the *PsEND1::barnase* plants. Segregation studies indicated that the inheritance and stability of the transgenes in the progeny were maintained in the next generation (data not shown).

SEM analysis of the male-sterile anther structures

The analysis of the cellular types present in the epidermis by SEM of the sterile anther structures (Fig. 5b, c and d) showed dramatic changes in shape, size and ornamentation when compared with WT anthers (Fig. 5a). The WT anther epidermis of both cultivars (Fig. 5e) showed a characteristic irregular cell type (toothed cells) with a peculiar ornamentation (Fig. 5f), whereas the sterile anther structures (Fig. 5h) showed a more regular and small cell type (Fig. 5i) with an ornamentation like that present in the cellular types of the anther filament (Fig. 5g, j). The cells forming the anther filament (Fig. 5j) in these plants are shorter than those present in the WT filaments (Fig. 5g), but their shape and ornamentation are quite similar.

In general, no pollen grains were found into the sterile anther structures and there was a notable reduction in the size of the middle layer, endothecium and connective tissues at each of the developmental stages examined. Only one of the transgenic lines (KB511.2), which showed a small structure similar to a pollen sac (Fig. 5d), had several undeveloped pollen grains (Fig. 5l). These abnormal pollen grains showed a collapsed shape when compared with the normal pollen grains present into the WT locules (Fig. 5k). Pollen viability and germination assays were negative for these abnormal pollen grains (not shown).

PCR analysis of the barnase transgene in the selected transgenic lines

The presence of the *barnase* transgene in the selected male-sterile lines of both cultivars was confirmed by PCR. The *barnase* gene was detected in all the transgenic plants analyzed (Fig. 4j). Only in the line KB511.2, the *barnase* band showed less intensity when compared with other transgenic lines and with the positive control (C+). This line also showed sterile anther structures with a less dramatic phenotype than the other ones (Fig. 5d) and produced a small amount of non-viable pollen grains (Fig. 5l).

Discussion

New or improved varieties of floricultural crops can be obtained via classical breeding by acting on floral traits, such as color, shape or fragrance, on vase life in cut-flower species and on rooting potential or overall plant morphology. In the case of the ornamental plant *K. blossfeldiana*, new traits with commercial interest have been introduced recently via genetic engineering (Christensen et al. 2008; Sanikhani et al. 2008; Topp et al. 2008; Thirukkumaran et al. 2009), however most of the transformation protocols reported previously showed low efficiency, high escapes rate and transgene silencing (Aida and Shibata 1996, 1998). We described here an efficient genetic transformation protocol applicable to different *K. blossfeldiana* cultivars. Shoot regeneration from leaf explants of *K. blossfeldiana* has been successfully achieved by employing IAA and kinetin (Smith and Nightingale 1979), and IAA and BA (Aida and Shibata 1996). Thidiazuron (TDZ) has been used to induce shoot regeneration in various *K. blossfeldiana* cultivars and addition of NAA did not improve regeneration, although a high variability was found among cultivars in terms of regeneration ability (Sanikhani et al. 2006). In the present work, the cultivars tested gave a very good response with NAA and BA for adventitious shoot formation and explants derived from axenic plants gave a superior regenerating rate compared to explants derived from greenhouse plants. Aida and Shibata (1996, 1998) studied the genetic transformation of the ‘Tetra Vulcan’ cultivar. Transformation efficiency was reported only in their first work, ranging from 1.4 to 2.9%. Using our protocol, transgenic plants of *K. blossfeldiana* were produced with significantly high transformation efficiencies, ranging from 11.2 to 75.8%. In the first two experiments, kanamycin was used at 25 mg l⁻¹ as selective agent. In spite of the successful regeneration of transgenic plants, a substantial proportion of escapes was identified. This was possibly due to a very efficient regenerating system, a low kanamycin dose, or a combination of both factors despite the regeneration experiments showing that 25 mg l⁻¹ kanamycin were enough to inhibit organogenesis. Aida and Shibata (1996) equally observed appearance of escapes (from 8.5 to 12.2% of explants) and used a low kanamycin dose for selection (20 mg l⁻¹) in the regeneration medium. High efficiency of regeneration may prompt some protective effect by transformed cells against non-transformed cells (Cervera et al. 1998). In order to solve this problem, in the experiments 3 and 4, the kanamycin dose was increased to 100 mg l⁻¹. The outcome was that escapes were reduced almost to zero and the transformation efficiency was higher. Transgene silencing was not a problem in our transformation system, contrary to what was reported by Aida and Shibata (1996).

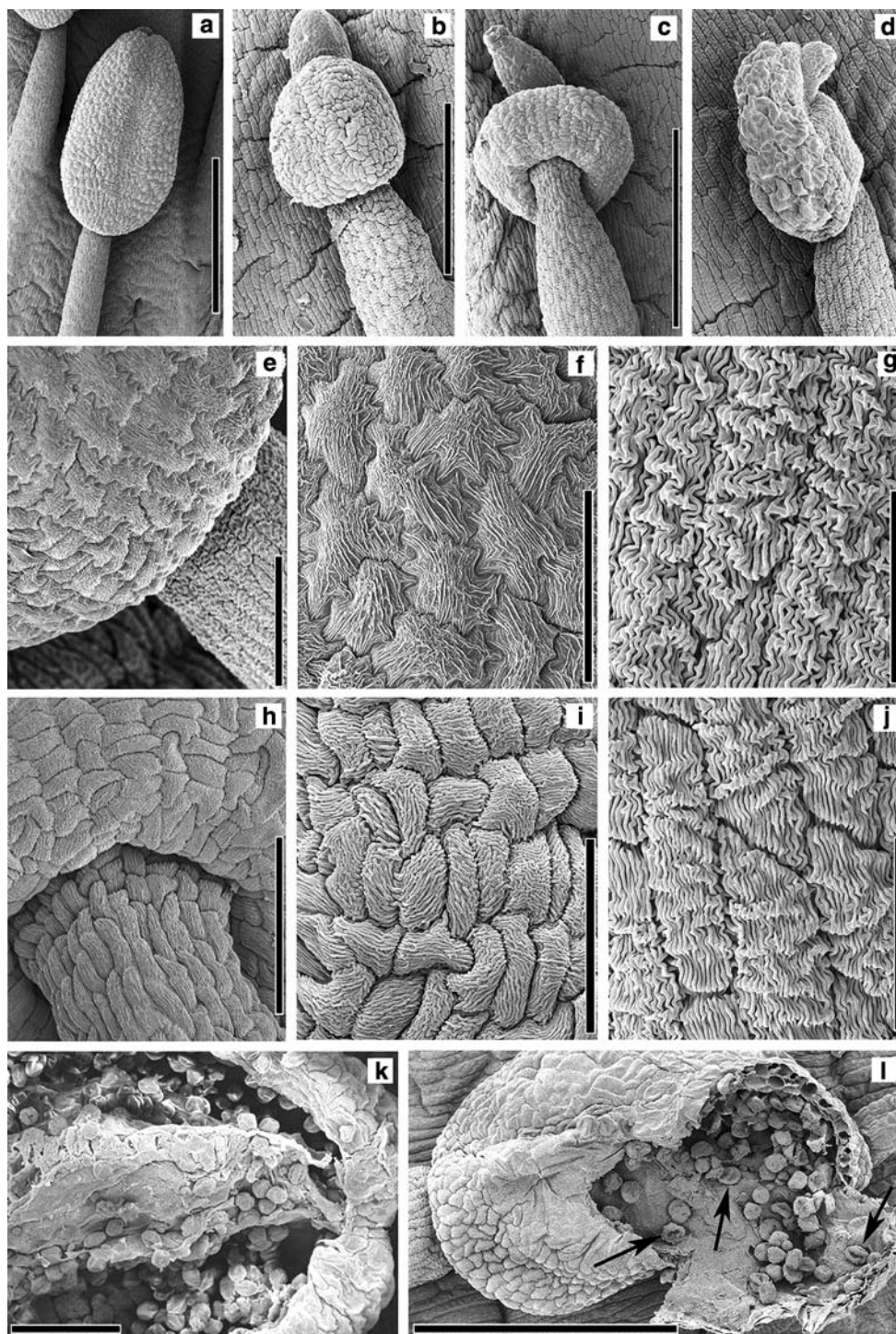


Fig. 5 Phenotypic analysis of male-sterile anthers by SEM. **a** WT stamen of a non-transformed plant showing the filament and the four-lobed anther. **b–d** Transgenic stamens from different male-sterile plants showing small structures at the end of the filament (*stick-like anthers*) in the place of a four-lobed anther. Epidermal cells show dramatic changes in shape, size and ornamentation when compared with WT anthers. **e** The WT anther epidermis of both cultivars showed a characteristic irregular cell type (*toothed cells*) with a peculiar ornamentation. **f** Close-up of the epidermal cell type in a WT anther. **g** Close-up of a WT filament showing the highly ornamented enlarged cells. **h** A sterile anther structure showing a more

regular and small cell type (non-toothed) with ornamentation like that present in the cellular types of the anther filament. **i** Close-up of the epidermal cells of a sterile anther. **j** The cells forming the anther filaments in these plants are shorter than those present in the WT filaments, but their shape and ornamentation are quite similar. **k** Section of a WT anther showing normal pollen grains into the locules. **l** Only in one of the transgenic lines (KB511.2), which showed a more developed pollen sacs, we detected the presence of several undeveloped pollen grains with a collapsed aspect (*black arrows*). Bars represent 800 μm in **a**; 400 μm in **c**; 300 μm in **b**, **d**, **l**; 100 μm in **e**, **k**; 80 μm in **f**, **h**, **i**; 30 μm in **g**

In genetic transformation experiments, the analyses generally focus on the molecular characterization of the transgenic plants but the ploidy state of the transgenic material is only checked in relatively few cases. The confirmation of the ploidy level in transgenic material is particularly important when a polysomatic tissue is used as the explant source. *K. blossfeldiana* cultivars with higher levels of ploidy have been obtained by inter-specific hybridization and modern cultivars have higher ploidy levels than the wild species (Van Voorst and Arends 1982). Aida and Shibata (2002) reported as well polyploid plants from transformation and regeneration experiments employing leaf as source tissue from a tetraploid cultivar ('Tetra Vulcan', $2n = 4x = 68$). From 116 regenerated plants, only 24 (20.7%) maintained the same ploidy level and the remainder had higher levels (8x, 12x and even 16x). Schwaiger and Horn (1988) using *Kalanchoe* hybrids reported many variants among regenerated plants; some of them had dwarf phenotypes and thick leaves and are hypothesized to be polyploid plants although ploidy level was not analyzed. Our results suggest that leaf tissues of cultivars used here have a polysomatic structure and regeneration from these explants leads to plants with increased ploidy levels. This does not exclude the possibility that duplication occurred during in vitro culture and/or the transformation phase.

Theoretically, *gfp* expression in transformed cells should be useful when selecting transformation events in early stages so that antibiotic selection is not needed. This gene has been used to transform successfully sugar cane, tobacco, maize, lettuce (Elliot et al. 1999), walnut (Escobar et al. 2000), citrus (Ghorbel et al. 1999), peach (Pérez-Clemente et al. 2004), potato (Rakosy-Tican et al. 2007), pear (Yancheva et al. 2006), carrot (Baranski et al. 2006) and other species (Mercuri et al. 2001). In *K. blossfeldiana* we have observed *gfp* expression in the regeneration process but we could not correlate explants with green fluorescence 3 weeks after inoculation with explants that regenerate transgenic plants. Low levels of *gfp* fluorescence are associated with increasing content of chlorophyll because chlorophyll red autofluorescence interferes with the *gfp* green fluorescence (Hraska et al. 2006). This fact could also be caused by pigments that are opaque to exciting UV or blue light (Mercuri et al. 2001) as it can happen in *K. blossfeldiana* petals, where no fluorescence is detected in the adaxial surface, which has a high content of floral pigments, while green fluorescence is clearly visible in the abaxial surface. Some authors state that transformation efficiencies based on resistance to a selective marker are probably underestimating the actual frequency of regenerated transgenic plants. Therefore PCR analysis is proposed to identify transgenic plants without the use of selectable or visual screening markers (Domínguez et al. 2004). In the present work, the *gfp* gene was useful to confirm genetic

transformation of *K. blossfeldiana*. All transgenic plants showed green fluorescence nearly in all tissues analyzed but there is a differential green fluorescence between organs and tissues.

Another important aspect of the present work is the production of engineered male-sterile plants of *K. blossfeldiana* to prevent undesirable gene flow of the introduced transgenes to related species. The *PsEND1* promoter specifically directed expression of the *barnase* gene to different anther tissues involved in anther architecture (epidermis, endothecium, middle layer, connective). Expression of barnase under control of this promoter caused specific ablation of these tissues at early stages of anther development in the transgenic plants. This was readily observed by the small structures developed instead of normal anthers in the third floral whorl, by the premature senescence and collapse of the pollen sac, microspores and tapetum and by the lack of pollen at anthesis in the transgenic flowers. Ablation of the structural anther tissues also produces the improper formation of the tapetum tissue and the subsequently degeneration of the microspores is accompanied by a change in anther wall thickness, by a size reduction and by a change in the epidermal cell types (Roque et al. 2007). Since this phenotype is unlikely due to expression of barnase in structural tissues, it is likely an indirect effect of the loss of the tapetum and microspore cells.

No viable pollen grains were observed (SEM) in any section of the ablated anthers from the male-sterile plants, indicating that barnase effectively ablates specific cell lines that will form the structural tissues of the anther, preventing pollen development. Transgenic anthers appear to show effects of barnase expression at every stage examined. This is likely due to the developmentally earlier expression of the *PsEND1* promoter. The anther filaments of the transgenic plants were short when compared with WT filaments. The formation of short filaments is commonly associated with male sterility or reduced fertility (Theis and Röbbelen 1990; Mariani et al. 1990; Denis et al. 1993; Worrall et al. 1992). These facts reinforce our previous results in other crop species using the same chimaeric construct (Roque et al. 2007).

Due to the extremely toxic nature of barnase, other researchers have reported a general lack of vigor and a decline in plant growth in plants carrying genes with barnase (Jagannath et al. 2001; Stanislaus and Cheng 2002; Wei et al. 2007). The lack of significant effects on growth characteristics is important to know when considering the use of barnase for male sterility in landscape plants. To prevent the effects of a possible ectopic barnase expression, Gardner et al. (2009) proposed the introduction of the male and/or female sterility genes in combination with a gene protecting against inappropriate barnase expression (enhanced *35S::barstar*). In all the lines of transgenic

Kalanchoe plants expressing barnase under control of the *PsEND1* promoter, we found no differences in vegetative growth, flowering time or inflorescence number. Morphological analysis of the transgenic plants showed that, under greenhouse conditions, the expression of the *PsEND1::barnase* construct does not significantly affect the vegetative and floral development, so confirming the anther specificity of the *PsEND1* promoter region previously observed by means of *uidA* expression studies in different dicots and monocots (Gómez et al. 2004; Pistón et al. 2008). The potential biotechnological applications of the *PsEND1* promoter largely depends on both its spatial and temporal expression pattern since the ectopic expression of the cytotoxic agent would damage other plant tissues and organs, decreasing the agronomic value of hybrid plants. The common characteristic of the *PsEND1::barnase* plants was the failure in producing fruits and seeds, as a consequence of the anther defects. Lack of detrimental effects of the sterility gene construct on plant growth and flowering is promising for future use of the *PsEND1::barnase* construct in combination with a female-sterility engineered system to create non-invasive landscape plants.

Expression of the *barnase* gene in ornamental plants under control of the anther-specific *PsEND1* promoter may be used to create efficient male-sterile versions of existing popular cultivars without adversely affecting the respective phenotypes. This would be especially useful to produce environmental friendly transgenic plants carrying new traits by preventing gene flow between the genetically modified ornamentals and related plant species and for ornamental species whose planting is discouraged due to their invasiveness. The invasiveness of ornamental crops is a problem that has attracted increased attention. Invasive plants pose a threat to the environment by displacing native plants, disrupting habitats and changing the balance of ecosystems. A significant number of the plants recognized to be invasive today were originally introduced as ornamental plants (Smith and Anderson 2006). With more non-native species being introduced on a continual basis for ornamental use, the identification of a system to prevent the introduction of plants with invasive potential becomes very important. One strategy to prevent ornamental crops from becoming invasive is to engineer them to be male and female sterile. This method is effective for reducing the invasive potential of species that set prolific amounts of seeds, which for land plants, is a primary determinant for invasiveness. Elimination of seed set would prevent many species from proliferating rapidly and spreading beyond the range where they were cultivated (Gardner et al. 2009).

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