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ORIGINAL PAPER

Expression of *KxhKN4* and *KxhKN5* genes in *Kalanchoë blossfeldiana* 'Molly' results in novel compact plant phenotypes: towards a cisgenesis alternative to growth retardants

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Abstract Many potted plants like *Kalanchoë* have an elongated natural growth habit, which has to be controlled through the application of growth regulators. These chemicals will be banned in the near future in all the EU countries. Besides their structural functions, the importance of homeotic genes to modify plant architecture appears evident. In this work, the full length cDNA of five *KNOX* (*KN*) genes were sequenced from *K.* x *houghtonii*, a viviparous hybrid. Two constructs with the coding sequence of the class I and class II homeobox *KN* genes, *KxhKN5* and *KxhKN4,* respectively, were overexpressed in the commercially important ornamental *Kalanchoë blossfeldiana* 'Molly'. Furthermore, a post-transcriptional gene silencing construct was made with a partial sequence of *KxhKN5* and also transformed into 'Molly'. Several transgenic plants exhibited compact phenotypes and some lines had a relative higher number of inflorescences. A positive correlation between gene expression levels and the degree of compactness was found. However, a correlation between the induced phenotypes and the number of inserted copies of the transgene were not observed, although line '70-10' with a high copy number also had the highest expression level. Moreover, overexpression of *KxhKN4* resulted in plants with dark green leaves due to an elevated content of chlorophyll, a highly desired property in the ornamental plant industry. These transgenic plants show that a cisgenesis approach towards production of compact plants with improved quality as an alternative to chemical growth retardants may be feasible.

Keywords Cisgenesis · Homeotic gene · Karyotype · KNOX · Plant architecture · Vivipary

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Introduction

Many *Kalanchoë* species like *K. blossfeldiana* and its interspecific hybrids are very important for the horticultural plant industry, and in 2009 *Kalanchoë* ranked as top one in Denmark with over 41 million plants produced (Rasmussen 2010). An important qualitative criterion for potted plants is that the produced plants should be compact; however, many potted plants like *Kalanchoë* have an elongated natural growth habit, which has to be reduced and controlled through the application of various growth retarding chemicals. Nevertheless, many of these compounds are potentially harmful to both human health and the environment (De Castro et al. 2004; Sørensen and Danielsen 2006; US Environmental Protection Agency 1993). In many countries several of these widely used compounds are no longer allowed (Fujimoto et al. 1997) and it is likely that more growth regulators will be banned in the near future in all the EU countries (Rademacher 2000). Several physical methods were proposed as substitutes for chemical growth retardants, e.g. using differences between day and night temperatures (DIF), cold morning, UV light, shaking

and brushing of plants, impedance and coloured nets to cover plants. Each of these methods also carries disadvantages (damage to plants, increased costs, low efficiency, and the problem of species or genotype specific responses) and, therefore, efficient and reliable protocols are still under development. From a breeders point of view, the genetic engineering approach offers an elite method to generate new variation when adequate natural diversity is absent in a given plant species. The *rol* genes from *Agrobacterium rhizogenes* have been used to obtain profusely flowering and compact plants in *Osteospermum ecklonis* and *Limonium* spp. (Giovannini et al. 1999; Mercuri et al. 2001) and recently it was shown that overexpression of *AtSHI* can produce compact *Kalanchoë* (Lütken et al. 2010). At present, a plethora of either plant transcription factors or structural genes that modify several aspects of plant architecture such as plant height, stem branching, inflorescence and flower morphology and flowering time, is reported in the literature (see Wang and Li 2006 for a review). Besides the role as structural genes (Carraro et al. 2006; Chary et al. 2008; WO/2007/148970; WO/2010/007497), the importance of homeotic genes to modify plant architecture appears evident (Yu et al. 2000; Barley and Waites 2002; Douglas et al. 2002; Sliwinski et al. 2007). Among the 14 classes of plant homeotic genes (Mukherjee et al. 2009), *BELL* and *KNOX* classes belonging to the TALE group, together with *WOX* (*Wushel related homeobox*) class play a central role in plant development being involved in meristem establishment, self maintenance and organ differentiation. *WOX* regulate early patterning events and tissue proliferation in *Arabidopsis* embryos (Haecker et al. 2004; Wu et al. 2007). *WOX2* and *WOX8* are co-expressed in the egg cell and zygote and later become confined to the apical and basal daughter cells of the zygote. *WOX2* is required for correct zygote development and in the adult plant the *Arabidopsis WUS* (*Wushel*) gene expression is restricted to a few cells below the uppermost layer of the L3 (rib zone) that generates the innermost layers of leaves and floral organs (Fletcher 2002; Bowman and Eshed 2000). WUS controls meristem function by direct regulation of cytokinin-inducible response regulators (Leibfried et al. 2005). TALE proteins, characterized by a three amino acid loop extension (proline-tyrosine-proline) in the loop connecting the first and second helices of the homeodomain, function as heterodimers and have evolved complex regulatory mechanisms controlling their subcellular localization (see Hamant and Pautot 2010 for review). The SKY and BELL domains of BEL1 proteins interact with the MEINOX domain of KNOX proteins and target the heterodimer to the nucleus (Bhatt et al. 2004; Cole et al. 2006). The *KNOX* gene family is divided into three classes (Hamant and Pautot 2010). Class I KNOX proteins contain a HWKPS peptide motif in the homeodomain (Kerstetter et al. 1994) and the genes are expressed mainly in the meristematic tissue. Class I KNOX proteins are represented by many members, e.g. *STM*, *BP*, *KNAT1*, *KNAT2*, *KNAT6* (*Arabidopsis*), *Knotted1* (maize) and *Bkn3* (barley). Class II KNOX proteins are characterized by the NWHSN motif (Kerstetter et al. 1994) and genes coding for these proteins are more widely expressed than class I genes and represented by *KNAT3*, *KNAT4*, *KNAT5*, *KNAT7* (*Arabidopsis*), *Zm_KNcl2a*, *Zm_KNcl2b* (maize), *MtKNcl2a*, *MtKNcl2b*, *MtKNcl2c* (*Medicago truncatola*). Class III proteins have a MEINOX domain but not a homeodomain and the unique *KNATM* gene member encodes a protein that selectively interacts with *Arabidopsis* BELL proteins through the MEINOX domain and dimerise with the KNOX protein BREVIPEDICELLUS (BP) through an acidic coiled-coil domain (Magnani and Hake 2008). Several members of the BELL family have been isolated in *Arabidopsis* (at least 13), in potato (*StBRL1 5, 11, 29* and *30*) and in *Trifolium* (*TpBEL1*-like). The *BEL1* gene directs normal flower development by interaction with *Agamous* (Ray et al. 1994), a homeotic gene that determines the C function in flowers. In addition to their specific function, members of the TALE family have overlapping and redundant functions. The last role has been correlated to the control of homeostasis of cytokinin and gibberellin (GA) (Shani et al. 2006). Activation of *KNOX* genes increases cytokinin biosynthesis. On the other hand, plants overproducing cytokinins have higher expression levels of *BP* and *SHOOT MERISTEMLESS* (*STM)* mRNA. *KNOX* genes negatively regulate GA biosynthesis via repression of GA-20 oxidase. In addition, cytokinins activate *WUS* and repress *CLAVATA* (*CLV1*), and both of these genes control meristem function and the number of stem cells in the meristem (Leibfried et al 2005; Gordon et al. 2009). Finally, auxin has a major role in downregulating *KNOX* expression during organ emergence (Hay et al. 2006).

Taken together, these data might indicate that homeobox genes, specifically the TALE group, may have a remarkable impact on ornamental plant architecture and hence attractiveness when overexpressed or downregulated. Moreover, alteration of these genes may, in a range of ways, help to simplify growing practices, production management costs, reduce impact on the environment and improve worker health.

In this work, we describe the cloning of *KNOX* (*KN*) genes from *Kalanchoë* x *houghtonii*. This plant, known as "Mother of Thousands", is a hybrid between *K. daigremontiana* and *K. delagoensis* and it develops epifillous plantlets on the leaf margin under long-day growth conditions. To obtain overexpression, two of the cloned genes, *KxhKN4* and *KxhKN5*, were transformed into *K. blossfeldiana* 'Molly' giving rise to the transgenic lines '69' and '70', and individual independent lines within, respectively.

Furthermore, an antisense construct of the *KxhKN5* gene was transformed into 'Molly' resulting in line '71'. The genetically modified *Kalanchoë* lines exhibited several horticulturally important phenotypes.

Materials and methods

Gene cloning

To identify *KNOX genes* involved in vegetative vivipary in the *K.* x *houghtonii* hybrid, leaf tissue was frozen before epifillous plantlet formation. Following RNA extraction using the RNeasy Plant mini kit (Qiagen, Hilden, Germany) and cDNA synthesis by Enhanced Avian HS RT-PCR (Sigma, St. Louis, MO, USA), semi-nested PCR was performed using an anchored oligo-dT primer and degenerated primers designed against a homeodomain sequence (Kobayashi et al. 2000). PCR products were cloned into the pGemTeasy vector (Promega; Madison, WI, USA) and sequenced. To identify full length coding sequence, nested 5′-RACE was carried out using whole or digested cDNAs adaptor libraries (Cottage et al. 2001). The partial sequences were aligned using the Clustal W multiple alignment tool and assembled using the contig tool of Bio Edit Software (Hall 1999). The complete coding sequences of each *K.* x *houghtonii* gene were compared to the GenBank database using the BLASTX algorithm (http://ncbi.nlm.nih.gov/BLAST/) and assigned to the *KNOX* gene classes I and II.

Phylogenetic trees

For the phylogenetic analysis, monocot and dicot representatives of KNOX I and II subgroups of proteins, with either known or unknown functions, were selected in the NCBI database together with the cloned *K.* x *houghtonii* genes. The deduced full length protein sequences were aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and phylogenetic analyses were carried out using the Phylip package v3.62 (http://evolution.genetics.washington.edu/phylip.html). Maximum likelihood methods were applied using the JTT model (Jones et al. 1992) of amino acid change and trees were visualized using TreeView v1.6.6 (http://taxonomy.zoology.gal.ac.uk/rod/treeview.html).

Constructs

To accomplish overexpression, the complete cDNA sequence of the genes *KxhKN4* (EU272790) (1,230 bp) and *KxhKN5* (EU240661) (1161 bp), overdriven by the *35S* promoter and NOS terminator, were cloned directionally into the *Eco*RI and *Not*I sites of the binary vector pGreenIINOSKAN (http://www.pgreen.ac.uk) that contains the *nptII* gene, conferring resistance to kanamycin. A post-transcriptional gene silencing (PTGS) construct was prepared by cloning a 326-bp fragment (bases 27–349) of *KxhKN5* into the specific cloning sites of pJM007 (Schattat et al. 2004) located at the left (*Not*I/*Bam*HI) and at the right (*Xba* I) of the *PIV2* intron. After screening for the correct insertion into the *Xba*I site, the silencing cassette was excised from pJM007 and cloned into the *Pst*I site of binary vector pGreenIINOS-KAN. This resulted in the overexpression vector constructs '69' and '70' containing *KxhKN4* and *KxhKN5*, respectively and vector construct '71' for silencing of *KxhKN5*. All constructs were transformed into *Agrobacterium tumefaciens* strain AGL1 (Lazo et al. 1991) containing pSoup (http://www.pGreen.ac.uk). Colonies were selected on LB plates containing rifampicin 100 mg l^{-1}, kanamycin 100 mg l^{-1}, tetracycline 10 mg l^{-1} and carbenicillin 100 mg l^{-1}. The constructs were verified by plasmid purification, restriction enzyme analysis and sequencing.

Plant material

Kalanchoë blossfeldiana cultivar 'Molly' (Knud Jepsen A/S, Hinnerup, Denmark) was established in vitro by sterilizing nodal cuttings for 15 min in 2% (v/v) NaOCl with 0.03% (v/v) Tween 20 (Sigma-Aldrich, Hercules, USA). Sterilization was done with gentle agitation (50 rpm) before the cuttings were washed thrice in sterile water. Explants were placed on standard MS medium (Murashige and Skoog 1962) with Gamborg vitamins (Duchefa, Haarlem, The Netherlands) (Gamborg et al. 1968), 3% (w/v) sucrose, 0.7% (w/v) bacto agar (basic medium) (pH was adjusted to 6.3 before autoclaving) in small plastic plant growth containers (5 × 6 × 9 cm) (Sakata Ornamentals Europe, Odense, Denmark) and allowed to form shoots. Shoots were cut off, rooted on basic medium and maintained in plastic containers. Explants and plants were cultured under a 16-h photoperiod of 45 μmol m^{-2} s^{-2} provided by cool-white fluorescent tubes (Philips, Eindhoven, The Netherlands).

Transformation of *Kalanchoë*

Transformation of *Kalanchoë blossfeldiana* 'Molly' was performed as described previously (Lütken et al. 2010). After 2 days co-cultivation on basic medium containing 15 mg l^{-1} acetosyringone, leaf discs were transferred to regeneration medium [basic medium supplemented with 1 mg l^{-1} thidiazuron (TDZ), 100 mg l^{-1} timentin (Tim100), 500 mg l^{-1} cefotaxime (Cef500), and 100 mg l^{-1} kanamycin (Kan100)]. All hormones and antibiotics were purchased from Duchefa (Saveen & Werner, Malmö,

Sweden). Leaf discs were transferred to fresh selective regeneration medium every third week. Shoots were placed in containers with basic medium containing Kan100, Tim100, Cef500. 0.85 mg l^{-1} of gibberellin (GA_3) and 1 mg l^{-1} of the auxin naphthalene acetic acid (NAA) (Sigma-Aldrich, Hercules, USA) was added to stimulate the formation of shoots and roots, respectively, when needed. Independent transgenic lines were established for each parental cultivar, and called overexpression of *KxhKN4* (lines '69') and *KxhKN5* (lines '70'), respectively, and *KxhKN5* antisense, *asKxhKN5* (lines '71'). Shoots from each transgenic line were rooted on peat in 10.5-cm pots at 16 h day/8 h night for 3 weeks, then placed at short day conditions (10 h day) for flower induction. During the day period, the light intensity was 150 µmol m^{-2} s^{-2}. Plants were grown in two independent randomized plots of ten plants.

Confirmation of transgenic plants by PCR

Genomic DNA was extracted from 400 mg leaves of regenerated plant tissue using the DNeasy Plant mini kit (Qiagen, Hilden, Germany). Transformants were verified by PCR with the specific primer sets shown in Table 1. The primers were designed by the Primer3 program (Rozen and Skaletsky 2000) using the specific gene sequences *KxhKN4* (EU272790); *KxhKN5* (EU240661); *KdActin* (GQ339777) and *nptII* from pGreenII0049 T-DNA sequence.

For the transgenic lines obtained using vector '69', '70' and '71', the following PCR program: initial denaturation at 95°C for 15 min and amplification of 35 cycles [(95°C for 30 s (denaturation), 55°C for 30 s (annealing), 72°C for 30 s (elongation)] followed by one cycle of 72°C for 7 min was used for detection of *KxhKN4*, *KxhKN5* and *asKxhKN5*, respectively. Presence of the *nptII* gene was verified by PCR to amplify a 0.7-kbp fragment using the following PCR conditions: one cycle of 95°C for 7 min, followed by 40 cycles of amplification (95°C for 1 min, 52°C for 1 min, 72°C for 1 min) followed by a final extension at 72°C for 7 min. PCRs were performed in a Veriti Thermal Cycler from Applied Biosystems (Foster City, CA, USA) and products were separated in 1% agarose gels, stained by GelRed (Biotium, Hayward, CA, USA) and visualized under UV light.

Relative copy number

Q-PCR was conducted as described by Lütken et al (2010), based on Bartlett et al (2008) using the *KN*-specific primers and *KdActin* as reference (Table 1). 100 ng DNA was used in each 25 µl PCR reaction and three replicates were carried out for each sample. Threshold cycles (C_t) (defined as the cycle where the signal exceeds ten times the standard deviation of the baseline), for *KN* genes were standardized to the *KdActin* C_t (=ΔC_t). The relative quantification of *KN* target genes in comparison to Wt was determined as $2^{-\Delta\Delta C_t}$ (Livak and Schmittgen 2001). PCR conditions were optimized for equal amplification efficiency between target genes *KN* and the housekeeping gene. Data from one out of two experiments, showing similar results, are presented.

Relative expression of the *KN* genes

Total RNA was extracted from young plant leaves and young shoots (approximately 1 cm in size) using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). Manufacturer's protocol was followed except for the lysis buffer; RLC buffer was chosen and supplemented with 3% (v/v) PEG 20.000 (Sigma-Aldrich, Steinheim, Germany) (Gehrig et al. 2000). Total RNA (1 µg) was DNase treated with the RQ1 RNase free DNase Kit (Promega, Madison, WI, USA) according to supplier's protocol and subsequently used for cDNA synthesis by the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's protocol. Real-time PCR was conducted as described above using the *KN*-specific

Table 1 Primer sequences used for detection of transgenic *KN* lines

Primer	Sequence	Primer position in the gene sequence (bp)	Fragment length (bp)
69f	5′-GCGGTATGGATTGCTCTGAT-3′	813	210
69r	5′-CCAGGCCTTCAAGTGAGAAG-3′	1,001	
70f	5′-GGCTGCAAGAGAGGAATTTG-3′	459	166
70r	5′-CATGAAATCCATCGCCTCTT-3′	605	
71f	5′-ATATGGAATTCCCAGCAGCA-3′	96	155
71r	5′-AATTGGGAAGTGGTGACAGC-3′	232	
KdActinf	5′-GCAGGACGTGATCTGACTGA-3′	289	168
KdActinr	5′-GACGGACGAGCTACTCTTGG-3′	437	
nptIIf	5′-GAGGTCTATTCGGCTATGACTG-3′	389	680
nptIIr	5′-ATCGGGAGCGGCGATACCGTA-3′	1,069	

primers and *KdActin* as reference (Table 1) and the amount of template in each reaction corresponded to 12.5 ng total RNA. Data from one out of two experiments, showing similar results, are presented.

Chromosome number determination

Plants for cytological investigations were grown in pots in a greenhouse (at 25°C). Root tips were harvested from well-growing plants, kept in iced tap water overnight, and then fixed at 20°C for 24 h in Clarke (3:1 abs. ethanol:glacial acetic acid) after which they were placed at −20°C for 48 h. For storage, root tips were transferred to 70% ethanol and kept at −20°C. Root tips for chromosome preparations were washed once in 5°C citrate buffer (0.0021% citric acid and 0.0029% tri sodium citrate dehydrate dissolved in demineralized water, adjusted to pH 4.6 and not autoclaved) and incubated in enzyme solution (citrate buffer added 2% cellulose, Onozuka RS and 10% pectinase, Sigma P-5146) for 12 min at 37°C. Root tips were then washed in 5°C citrate buffer for 30 min. On a clean microscope slide, root tips were placed individually in a drop of 45% acetic acid. The terminal 0.2 cm of the root tip was dissected; loose lying cells were covered by a cover slip, warmed up over an alcohol flame and gently tapped using a match. The slide was reheated gently and then squashed with a rolling thumb. Some preparations were stained in DAPI (4′,6-diamidino-2-phenylindole) solution (1 μg μl^{-1}) for 5 min and mounted in citifluor (Citiflour Ltd.) before examination with a Zeiss Axioskop with appropriate filters for DAPI. Other preparations were examined unstained in a Nicon Eclipse E 400. From each accession approximately three slides were made, each slide providing around 10(-3-5) counts.

Plant trait analysis

The effects of overexpressing and silencing the *KN* genes in *Kalanchoë blossfeldiana* 'Molly' were analyzed by scoring a range of specific plant quality traits: number of weeks until opening of first flower and appearance of first wilted flower, plant height and broadest plant diameter, and number of flower inflorescences. The plant quality data represent one out of two independent experiments with similar results.

Chlorophyll analysis

Chlorophyll *a* (Chl *a*) and *b* (Chl *b*) were extracted by incubation of leaf pieces of 63.5 mm^2 harvested with a cork borer from the third youngest leaf pairs (the exact weight was noted) in 1.5 ml 96% (v/v) EtOH at 80°C and determined after the leaves were completely devoid of pigments. Chl *a* and Chl *b* were then measured at wavelengths of 648.6 and 664.2 nm by the method of Lichtenthaler (1987). For each plant line, ten leaf pieces were harvested.

The relative content of chlorophyll was measured by a CL-01 chlorophyll content meter (Hansatech Instruments, King's Lynn, Norfolk, England) on both of the third youngest leaf pairs ($n = 15$). This method is based on dual wavelength optical absorbance (620 and 940 nm, respectively) measurements providing an indicator of photosynthetic activity relating to the nitrogen concentration of the sample. The relative chlorophyll content is displayed in the range of 0–2,000 units. The chlorophyll data represent one out of two similar measurements.

Statistical analysis

Plant traits and chlorophyll content data were statistically analyzed using the Microsoft Excel package. Significant differences were calculated using Student's *t* tests. Significant differences ($P \leq 0.05$) are indicated by asterisks in the figures.

Results and discussion

Isolation of *KxhKN* class I and II KNOX genes

The cDNA of four *knotted*-like genes belonging to class II (*KxhKN1* to *KxhKN4*) and one gene belonging to class I (*KxhKN5*) were identified in *K.* x *houghtonii* and their sequences were submitted to GenBank (NCBI): EU272787, EU272788, EU272789, EU272790, EU240661, respectively. The class I and class II KNOX proteins are characterized by the peptide motif 'HWKPS' and 'NWHSN' in the homeodomain, respectively (Fig. 1). Moreover, the characteristic domains of KNOX proteins (KNOX1, KNOX2, GSE and ELK) were identified in all sequences.

Phylogenetic analysis

The phylogenetic analysis of KxhKN5 (Fig. 2, Online Resource 1) showed that the inferred protein sequence clusters with several KNAT1 like proteins of dicot species, such as KNAP1, KNAP2 from *Malus domestica*, PpKNlike (KNOPE) from *Prunus persica* and PtKNlike from *Populus tomentosa*. Although, incomplete information about these genes has been published, *KNAP1* and *KNAP2* transcripts seem to be absent in the leaves and in floral organs, but they are expressed in the stem (Watillon et al. 1997). The KNOPE protein shows high homology to KNAP1/2 and shares the competence in maintaining the meristematic identity of cambium cells. KxhKN5 group is closely related

Fig. 1 Protein alignment of the homeobox domain of class I (*top*) and class II KNOX (*bottom*) proteins. The proteins were aligned by the ClustalW method. Accession numbers: KNAT1: NP_192555.1; KNAT2: NP_177208.2; STM: NP_176426.1; Kn1: AAY57559.1; KxhKN5: EU240661; OSH 45: BAA08553.1; KNAT3: NP_197904.1; KNAT4: NP_196667.2; KxhKN1: EU272787; KxhKN2: EU272788; KxhKN3: EU272789; KxhKN4: EU272790

to NTH2, NTH3, NTH20 from *Nicotiana tabacum* and TKN1 from *Lycopersicon esculentum* with uncertain functions (Fig. 2). Also KNAT1 (BP) from *Arabidopsis* and ChBRVDP from *Cardamine hirsuta* group in the same cluster, and share 71 and 70% identity with KxhKN5, respectively. *KNAT1* is expressed in the shoot apical meristem and stems and when it is ectopically expressed it causes lobed leaves and meristem development on either leaf vein or sinus regions (Chuck et al. 1996). In *C. hirsuta*, class I *KNOX* genes delay cellular differentiation and produce a dissected leaf form (Hay and Tsiantis 2006). The monocot proteins Bkn3 from barley and Knotted1 from maize belong to a more distant phylogenetic branch that only contains sequences from Poaceae. Bkn3 is responsible for the *Hodded* mutation in lemma (Müller et al. 1995) and both trigger shoot formation on the leaf surface when overexpressed in tobacco (Lin and Müller 2002; Sinha et al. 1993). A further major branch of class I tree group proteins less closely related to KxhKN5 as STM from *Arabidopsis* (Long et al. 1996) and several STM homologues, were involved in maintenance of meristematic cells in the shoot apical meristem (Fig. 2).

The phylogenetic analysis of class II *K.* x *houghtonii* proteins showed that KxhKN1, KxhKN3 and KxhKN4 cluster in a group closely related to LET12, solkn2 and LycescH1 all from *Lycopersicum esculentum*, whereas KxhKN2 seems more distant from them (Fig. 3, Online Resource 2). The function of class II *KNOX* genes is yet poorly understood. *LET12* seems ubiquitously expressed in the mature plant (Janssen et al. 1998). *NTH23* from *Nicotiana tabacum* that is related to *KxhKN2* is expressed in the basal region of leaf primordia and may be important for the lateral growth of leaf blades (Sentoku et al. 1998).

Regeneration of transgenic lines

Two overexpression vector constructs with *KxhKN4* ('69') and *KxhKN5* ('70') and an antisense construct with *asKxhKN5* ('71') under the control of the *35S* promoter were introduced into the *K. blossfeldiana* 'Molly' by *Agrobacterium tumefaciens*-mediated transformation. This gave rise to transgenic lines using the vector number as prefixes '69-', '70-' and '71-', respectively.

The transformation efficiency was close to 50% for all constructs, determined by the plants ability to root on kanamycin containing medium and their maintenance of leaf chlorophyll. From an early phase in the differentiation of callus, distinct phenotypes appeared in the transgenic lines. Transgenic '69-' lines appeared more dark green compared to the untransformed control, whereas '70-' lines had hand flapped leaves, with marked lugs, compared to the untransformed control. Independent transgenic lines were selected for each vector construct for further phenotypical evaluation.

Molecular analysis

Presence of the specific *K.* x *houghtonii KN* genes was verified by PCR using gene-specific primers in each transgenic line. These primers do not recognize any endogenous *K. blossfeldiana KN* genes (Fig. 4, lane 'Molly'). For the two overexpression constructs '69' and '70', five transgenic lines were selected for further analysis while four transgenic lines were selected for the antisense construct '71'.

The relative copy number was analyzed by qPCR according to the method described by Bartlett et al. (2008) and Lütken et al. (2010). The relative copy numbers of the

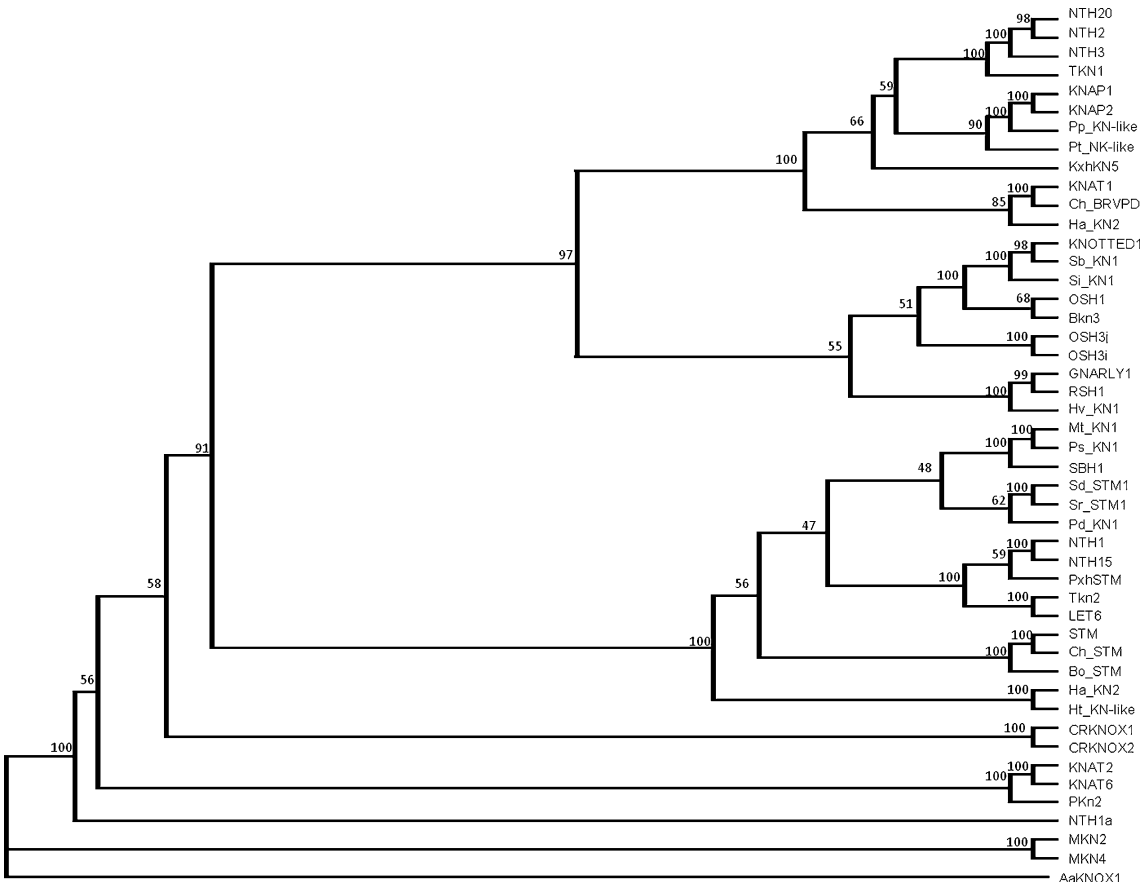

Fig. 2 Phylogenetic tree of the class I *K.* x *houghtonii* KN5 protein and selected relatives from other plant species. Full length sequences were used for the alignment. Species of origin, accession number and description of each protein are given in Online Resource 1. *Numbers* at the branches indicate bootstrap values for 100 trials

KN genes inserted into the plant genomes were calculated by standardizing the *KN* presence to the presence of *KdActin*. The *KN* lines were tentatively categorized into three tentative groups: single copy (<1.75-fold change), low copy number (1.75–3.5-fold change), and high copy number (>3.5-fold change). All the '69' lines were classified as single copy lines as well as lines '70-2', '70-22' and '71-F' (Fig. 5). Line '70-23', '70-6', '71-1', '71-14' and '71-18' were assigned as low copy number lines whereas line '70-10' showed many copy of the transgene (Fig. 5). A similar distribution of tentative copy numbers was found when *AtSHI* was ectopically expressed in *Kalanchoë* 'Molly' (Lütken et al. 2010).

The expression of the *KN* genes in leaves was investigated by quantitative real-time RT-PCR by correlating *KN* transcript levels to endogenous *KdActin* levels and then comparing these values between *KN* lines and Wt plants. Both *KxhKN4* and *KxhKN5* overexpressing lines that displayed the most extreme phenotypes typically (Fig. 6a, b) also had the highest gene expression levels in the leaves (Fig. 5). Expression of *KxhKN5* was not detected in any of the antisense lines confirming either a undetectable expression or an early degradation of the coded mRNA. The *KN* gene expression levels were further investigated in stems to compare expression levels with respect to tissue specificity. For all lines, the *KN* gene expression levels in stems were found to be comparable to the expression levels in leaves (data not shown).

Comparison of the tentative *KN* gene copies number within the transgenic lines and the gene expression levels did not indicate a correlation, although line '70-10' with a high copy number also had the highest expression level (Fig. 5).

Plant phenotypes

All of the transgenic *KN* lines exhibited compact phenotypes usually with reduced plant height, and many lines differed significantly in comparison to the Wt control (Fig. 6). Moreover, a common feature for all of the transgenic lines harboring a specific gene construct was that the individual transgenic lines showed a range of dwarfing phenotypes. For both '69-' and '70-' transgenic lines, this means that the shortest lines were approximately only half

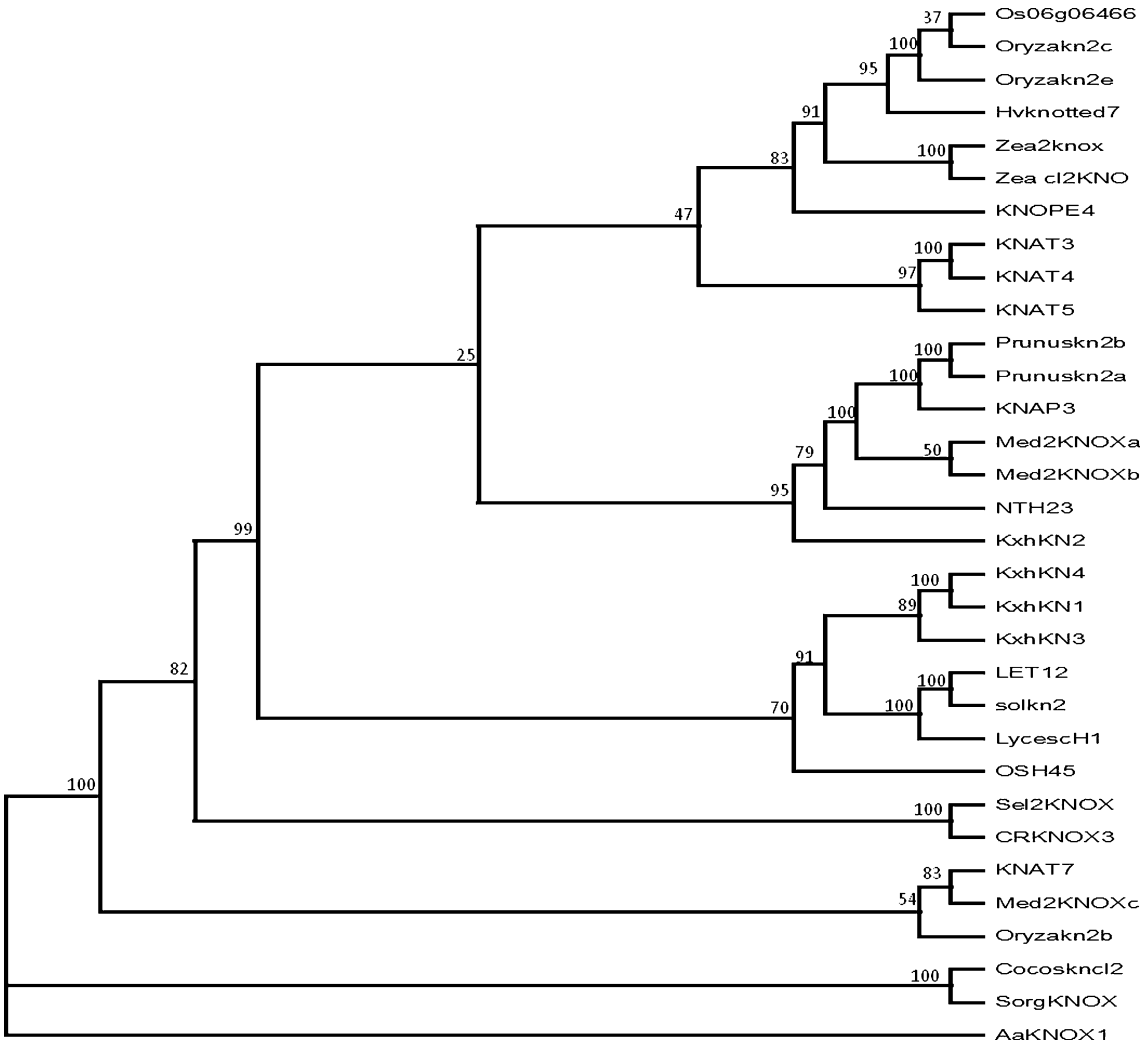

Fig. 3 Phylogenetic tree of four class II *K*. x *houghtonii* proteins (KN1 to KN4) and selected relatives from other plant species. Species of origin, accession number and description of each protein are given in Online Resource 2. *Numbers* at the branches indicate bootstrap values for 100 trials

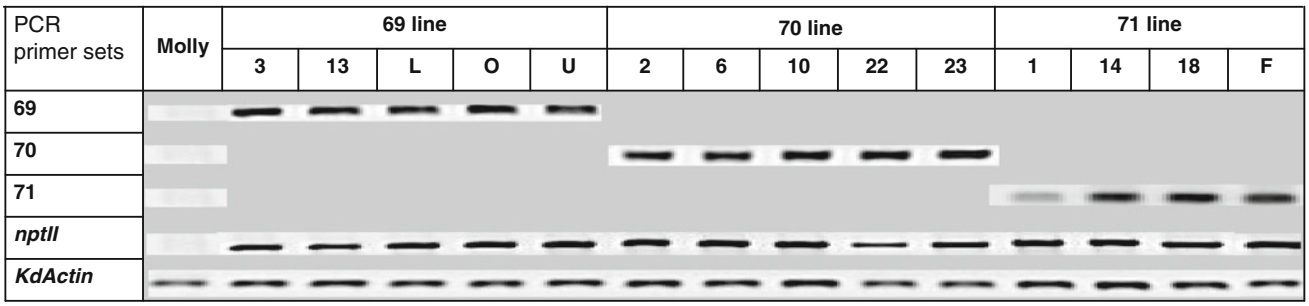

Fig. 4 Identification of transgenic *Kalanchoë KN* lines ('69', '70' and '71') by PCR. Wt 'Molly' served as control. The reactions were analyzed by agarose gel electrophoresis and the respective GelRed stained PCR products are shown

the height of the tallest (Fig. 6a, b). Specific morphological features characterized all the lines harboring a specific gene construct. All the transgenic '70' lines exhibited altered leaf morphology and a very small plant diameter (Fig. 6b, d, e). Among '71' lines carrying the antisense construct, only line '71-F' exhibited altered leaf morphology (Fig. 6c, d) and looked similar to many of the lines transformed with the '70' construct. None of the '69' transgenic lines showed

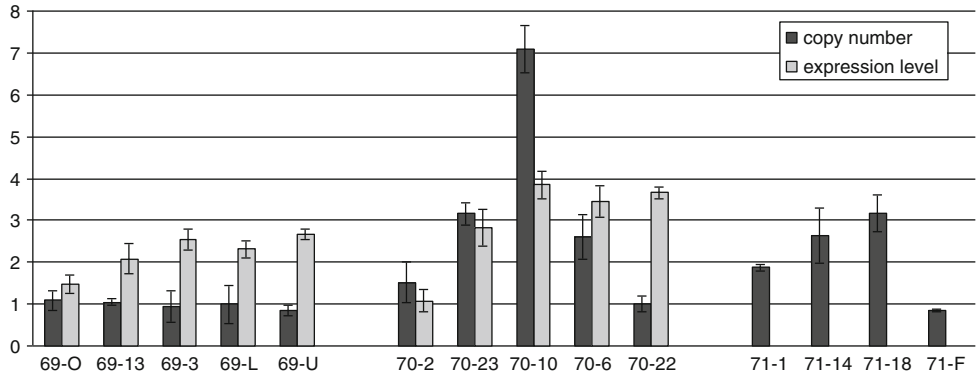

Fig. 5 Tentative *KN* copy numbers and expression levels. The values were calculated as fold change $2^{-\Delta\Delta C_t}$ for *KN* standardized to the *KdActin* threshold cycles (ΔC_t) with the differences between the *KN* lines and Wt calculated as $\Delta(\Delta C_t)$. The tentative copy number groups were: single (<1.75-fold change), low (1.75–3.5 fold change), high (>3.5-fold change). *Bars* represent averages ± standard deviation. The data represent one out of two independent experiments, where similar results were obtained

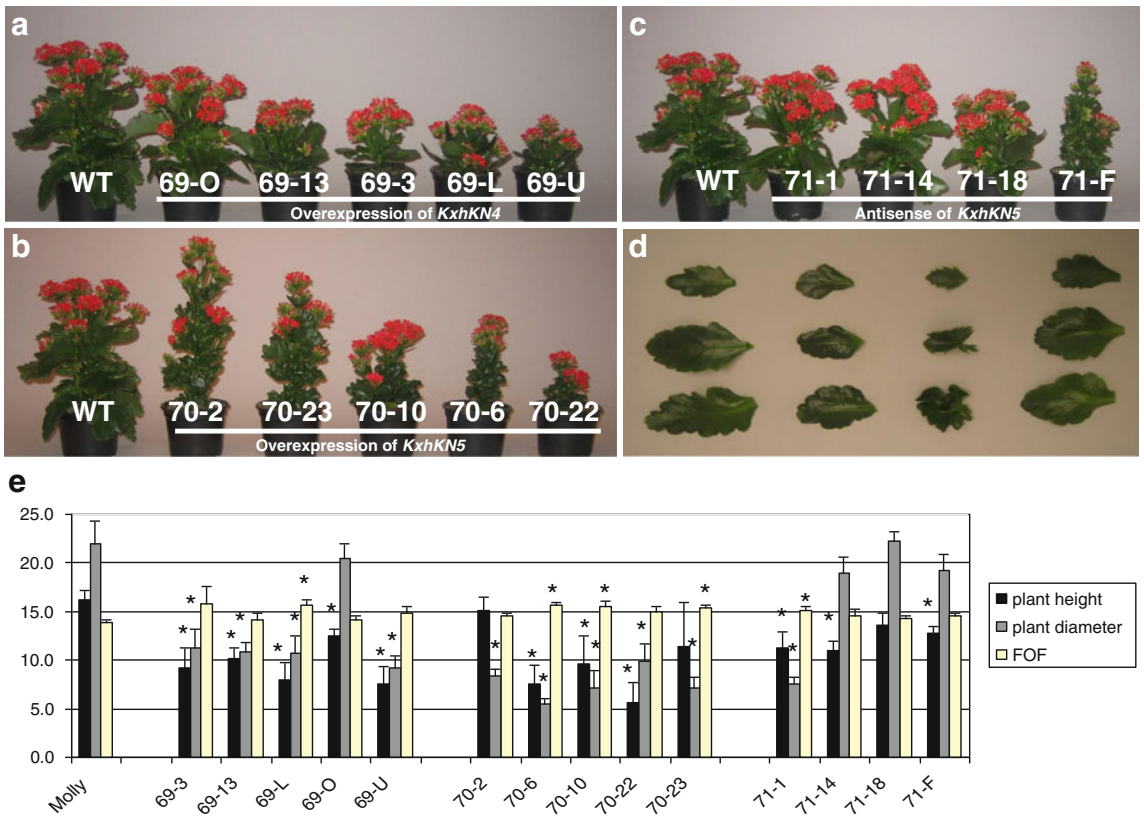

Fig. 6 Plant phenotypes and biometrical analysis. **a** *KxhKN4* overexpression lines. **b** *KxhKN5* overexpression lines. **c** *KxhKN5* antisense lines. **d** From *left* to *right* leaves of 'Molly' Wt control plant, '69-13', '70-23' and '71-14'; the *lower row* shows ground leaves, the *middle row* shows leaves supporting the inflorescences and the *upper row* shows leaves from the inflorescences. **e** biometrical data for plant height and plant diameter are shown in cm, while first open flower (FOF) are counted weeks from planting. Plants were grown in two independent randomized plots of ten plants. Values are shown with standard deviations and *asterisks* indicate values significantly different from Wt ($P \leq 0.05$). The data represent one out of two independent experiments, where similar results were obtained

leaf alteration (Fig. 6a, d). With the exception of line '71-F', the '71' lines had almost twice the plant diameter of the '70' lines.

A compact inflorescence consisting of many branches and high numbers of branches per unit of stem are an important quality trait in horticulture (Oerum and Christensen 2001). The number of inflorescence branches was counted in the transgenic lines and although it was not significantly higher (data not shown), the line '69-O' had a significantly higher number of branches per cm of stem

Table 2 Number of branches in transgenic lines '69-O' and '71-18' in comparison to 'Molly' Wt

Line	'Molly' Wt	69-O	71-18
Number of branches	12.4 ± 1.9	13.6 ± 1.1	12.2 ± 0.9
Number of branches per cm	0.77 ± 0.15	1.03 ± 0.08*	0.90 ± 0.12

The number of branches per cm was calculated by dividing the number of branches with the total plant height. Values are followed by standard deviations and asterisks indicate values significantly different from Wt ($P \leq 0.05$)

Table 3 Total and relative chlorophyll content in transgenic *KN* lines

Chlorophyll content	'Molly' Wt	69-3	69-13	69-L	69-O	69-U
Total μg $mgFW^{-1}$	0.42 ± 0.04	0.55 ± 0.08*	0.63 ± 0.07*	0.64 ± 0.11*	0.43 ± 0.14	0.55 ± 0.6*
Relative units	16.8 ± 2.8	53.1 ± 17.0*	37.2 ± 7.1*	53.9 ± 14.5*	28.4 ± 5.6*	37.2 ± 12.9*

Values are followed by standard deviations and asterisks indicate values significantly different from Wt ($P \leq 0.05$)

(Table 2). We have previously shown that overexpression of the zinc finger transcription factor *short internodes* (*SHI*), isolated from *Arabidopsis*, which reduces gibberellin response, resulted in compact *Kalanchoë* (Lütken et al. 2010). However, in that study none of the transgenic lines had a higher number of inflorescence branches.

Delayed flowering increases the production cost in the ornamental plant industry. Although some transgenic lines were delayed 1–2 weeks in flowering, the majority of the lines flowered as early as the 'Molly' Wt (Fig. 6e). Hence, selection for early flowering among transgenic lines can bypass a possible pleiotropic negative effect caused by the transgenes. The duration of the flowering period lasted 5–6 weeks for all transgenic lines and no significantly different changes were observed compared to Wt 'Molly' (data not shown). An unaltered flowering period was also found when *AtSHI* was overexpressed in *Kalanchoë* 'Molly' (Lütken et al. 2010).

It has previously been shown that *Agrobacterium*-mediated transformation of *Kalanchoë* can lead to increased ploidy levels, and that high ploidy levels typically result in compact plants (Aida and Shibata 2002). The ploidy levels in the transgenic *KN* lines were determined by counting the chromosome numbers, and due to the polysomic nature of *Kalanchoë* (Izumikawa et al. 2008) efforts were made on determining the highest chromosome numbers observed within the transgenic lines. 'Molly' is a tetraploid cultivar ($2n = 4x = 68$) (Kai Lønne, personal communication) of the diploid *K. blossfeldiana* species ($2n = 2x = 34$) (Aida and Shibata, 2002). Except for line '69-3' that exhibited an increased number of chromosomes ($2n \sim 110$–120), all lines had chromosome numbers comparable to 'Molly' Wt. (Online resource 3). This suggested that *KN* gene expression levels instead of an altered ploidy level triggered the compact phenotypes.

The dark green color of the leaves detected in all '69' lines (Fig. 6a) was investigated further by determining the total concentration of chlorophyll in the leaves (Lichtenthaler 1987). These data indicated that several of the transgenic lines had significantly higher content of chlorophyll. Lines '69-13' and '69-L' with around 0.64 μg $mgFW^{-1}$ chlorophyll had almost 50% more chlorophyll compared to the 'Molly' Wt which contained 0.42 μg $mgFW^{-1}$ chlorophyll (Table 3) similarly to other *K. blossfeldiana* cultivars like 'Rako' (Hwang et al. 2008). The relative content of chlorophyll was measured by CL-01 chlorophyll content meter (Hansatech Instruments, King's Lynn, Norfolk, England) that measures photosynthetic activity in relation to the nitrogen concentration in the sample. For some lines, there was a correlation between the two methods of chlorophyll determination (Table 3) Correlation between the two methods has previously been shown in other plant species (Cassol et al. 2008). Some contrasting results can be expected when two different types of chlorophyll measurements are compared, but taken together both measurements indicated a higher chlorophyll content in the '69' lines. The dark green leaf color is an important property in floriculture, as it is favored by the consumers (Townsley-Brascamp and Marr 1995), hence overexpression of *KxhKN4* adds further ornamental value to the compact '69' lines.

Overexpression of *KxhKN5* genes clearly affected leaf morphology (Fig. 6d) and this feature has been demonstrated previously by Lincoln et al. (1994), where *KNAT1* from *Arabidopsis* was overexpressed in transgenic plants. Both genes belong to the same major branch of the class I phylogeny tree (Fig. 2). Although the leaf morphology was not altered by overexpression of *KxhKN4*, all the lines exhibited a reduction in leaf size (Fig. 6d). This was also the case with antisense of *KxhKN5*, with the exception of line '71-F', which phenotypically looked similar to the *KxhKN5* overexpression lines. As mentioned above, expression of *KxhKN5* was not observed in any of the '71' antisense lines. However, when *KxhKN5* was endogenously overexpressed

and silenced in *K* x *houghtonii*, a substantial variation was also observed in the leaf morphology between different lines (Laura et al. 2009). Based on these observations, it can also be expected that dissimilarities in leaf morphology can arise from antisense of *KxhKN5*.

In the present study, a single cultivar of *Kalanchoë* ('Molly') was transformed resulting in a dwarf phenotype for the majority of the lines. In a recent study (Lütken et al. 2010), it was shown that overexpression of *AtSHI*, in cultivar 'Molly' produced the least compact phenotypes out of the nine cultivars tested. Although these results may appear contradicting, they can be explained considering the different pathways involved. *KNOX* genes interact with several homeobox genes and play a role in hormone homeostasis (Yanai et al. 2005) with pronounced effects in many aspect of plant architecture, while *AtSHI* gene confers a more specific function determining a defect in gibberellic acid perception. Results from this work combined with those of intraspecific expression of *KxhKN5* in *K*. x *houghtonii* published earlier (Laura et al. 2009) allow us to suggest that modulation of both class I and class II *KNOX* genes serves as a tool to develop compact plants of *Kalanchoë* with a high horticultural value. Genetic variation exceeding those observed in primary transformed plants will be expected in self-fertilization and cross-derived progenies due to the rearrangement of transgene copy and transgene interaction with a new genomic background. Moreover, as the genes used in this experiment were isolated from a species within the same genus, overexpression of endogenous *KN* genes may be useful in a cisgenesis approach to develop stable compact plants as an alternative to chemical growth retardants. Intragenic and intraspecific regulatory promoter sequences that mimic 35S promoter may facilitate a full cisgenic approach with the production of a wider pattern of expression.

As the *KxhKN4* and *KxhKN5* genes originally were cloned from leaf tissue exhibiting vivipary, it might be expected that overexpression of the genes could trigger the formation of plantlets in species like *K. blossfeldiana* that do not form plantlets. However, we did not observe any plantlets forming on any of the transgenic lines even under a longer photoperiod that usually triggered plantlet formation in *K*. x *houghtonii* (Fig. 6.). Moreover, overexpression of *KxhKN5* in *K. x houghtonii* did not result in any ectopic formation of plantlets as well (Laura et al. 2009).

Conclusion

Homeotic genes are important in modifying plant architecture. In the current study, the two *KNOX* (*KN*) genes *KxhKN4*, *KxhKN5* isolated from *K*. x *houghtonii* were overexpressed and silenced (with a PTGS construct containing a partial sequence of *KxhKN5*) in the commercially important ornamental *K. blossfeldiana* 'Molly'. Collectively, overexpression of *KxhKN4* and silencing of *KxhKN5* resulted in compact phenotypes with reduced plant height and diameter and some lines even had a relative higher number of inflorescences. A positive correlation between gene expression levels and the degree of compactness was found, but no correlation was observed between the induced phenotypes and the number of inserted copies of the transgene. Compact plant habit (reduced plant height and diameter) together with dark green leaves and flowering time comparable to the 'Molly' Wt are traits of great ornamental value accumulated in '69' lines. The data further illustrate that a cisgenesis approach towards production of compact plants with improved quality as a stable alternative to hazardous growth retarding chemicals may be feasible.

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Conflict of interest The authors declare that they have no conflict of interest.

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