Plant Biotechnology Journal

Plant Biotechnology Journal (2010) **8**, pp. 211–222 doi: 10.1111/j.1467-7652.2009.00478.x

Production of compact plants by overexpression of *AtSHI* in the ornamental *Kalanchoë*

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Received 28 July 2009;
revised 24 September 2009;
accepted 22 October 2009.
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Summary

Growth retardation is an important breeding aim and an essential part of horticultural plant production. Here, the potential of transferring the *Arabidopsis* short internode (*shi*) mutant phenotype was explored by expressing the *AtSHI* gene in the popular ornamental plant *Kalanchoë*. A 35S-*AtSHI* construct was produced and transferred into eight genetically different cultivars of *Kalanchoë* by *Agrobacterium tumefaciens*. The resulting transgenic plants showed dwarfing phenotypes like reduced plant height and diameter, and also more compact inflorescences, as a result of increased vegetative height. The *shi* phenotype was stable over more than five vegetative subcultivations. Compared with *Arabidopsis*, the ectopic expression of *AtSHI* in *Kalanchoë* showed several differences. None of the *Kalanchoë* SHI-lines exhibited alterations in leaf colour or morphology, and most lines were not delayed in flowering. Moreover, continuous treatment of lines delayed in flowering with low concentrations of gibberellins completely restored the time of flowering. These features are very important as a delay in flowering would increase plant production costs significantly. The effect of expression controlled by the native *Arabidopsis SHI* promoter was also investigated in transgenic *Kalanchoë* and resulted in plants with a longer flowering period. Two *AtSHI* like genes were identified in *Kalanchoë* indicating a widespread presence of this transcription factor. These findings are important because they suggest that transformation with the *AtSHI* gene could be applied to several species as a tool for growth retardation, and that this approach could substitute the use of conventional chemical growth regulation in plant production.

Keywords: dwarf, growth retardants, *Kalanchoë,* ornamental, *Short Internodes,* transgenic plants.

Introduction

Growth retardation, to produce desired compact plants, is a financially important part of horticultural plant production (Oerum and Christensen, 2001). At present, growth retardation is accomplished by the use of various chemical growth regulators, but many of these compounds are potentially hazardous to the environment and to human health (US Environmental Protection Agency, 1993; De Castro *et al.*, 2004; Sørensen and Danielsen, 2006). The use of the two compounds paclobutrazol and daminozide for controlling growth are prohibited in several countries, and new temporary guidelines for their use have recently been released in Denmark (Grøn Plantebeskyttelse ApS, 2008) while the Environmental Protection Agency is reevaluating the use of these two chemicals. Growth retarding chemicals inhibit the synthesis of gibberellic acid (GA) (Rademacher, 2000), however, GA also influences flowering time, fertility and morphogenesis in general (Fleet and Sun, 2005).

Biotechnological approaches to achieve growth retardation have similarly focused on GA biosynthesis. In rice

(*Oryza sativa*), the 'green revolution rice' (Hedden, 2003; for review see Salamini, 2003), described as a semi dwarfed plant with significantly increased crop yield, has subsequently been shown to have a mutant allele of a *GA20-oxidase* (*GA20ox*), the gene encoding a rate-limiting enzyme in the active GA biosynthesis pathway (Lange, 1998).

Antisense silencing of *GA20ox* expression has been pursued in several plant species (Coles *et al.*, 1999; Carrera *et al.*, 2000; Oikawa *et al.*, 2004). In general, reduction of *GA20ox* expression produces dwarfed plants with reduced internode length and a reduction in the content of active GA. However, lowering the GA levels also affects other fundamental processes in the plant life cycle. In *Arabidopsis,* antisense silencing of *GA20ox*'s resulted in various phenotypes displaying smaller leaves, delayed flowering time and reduced fertility (Coles *et al.*, 1999). Although the effect on flowering could be rescued by GA application, this treatment also reversed plant height to wild type. Overexpression of genes controlling the inactivation of GA has also been shown to result in dwarfed plants with delayed flowering time (Biemelt *et al.*, 2004; Curtis *et al.*, 2005; Dijkstra *et al.*, 2008). We have previously shown that down-regulation of *GA20ox*'s under the control of an inducible promoter in *Kalanchoë* resulted in similar phenotypes (Topp *et al.*, 2008).

In recent years, several regulatory genes involved in GA signalling have been identified (recently reviewed in Hirano *et al.*, 2008). Studies of the GA response in *Arabidopsis* have unravelled a range of new genes involved in GA signalling, but have also revealed the presence of a complicated regulatory network of proteins with a high degree of functional redundancy. Many of the identified genes belong to a family of transcriptional regulators, the so called DELLA proteins (Eckardt, 2002; Wen and Chang, 2002) a family consisting of five genes in *Arabidopsis* (Dill and Sun, 2001). DELLA proteins function as negative regulators of the GA response and are rapidly degraded by the ubiquitin proteasome pathway when GA levels rise. Plant growth manipulation has also been attempted through expression of DELLA proteins, for example ectopic expression of the *Arabidopsis gai* (*GA INSENSITIVE*) mutant *DELLA* gene (Koornneef *et al.*, 1985) in rice conferred a dwarfed phenotype similar to the 'green revolution rice' (Fu *et al.*, 2001). Genetic analysis has determined that *gai* is a mutant DELLA gene resulting in a deletion of the DELLA motif DELLAVLGYKVRSSEMA giving the product increased potency because it is resistant to GA mediated degradation (Peng *et al.*, 1997). Ectopic expression of *gai* in *Chrysanthemum* also produced dwarfed plants. However, both the number and size of the flowers were reduced and the transgenic plants had delayed flowering time (Petty *et al.*, 2001). Furthermore, overexpression of the *Arabidopsis gai* gene in apple also reduced the plant size, but the transgenic clones showed reduced rooting ability (Zhu *et al.*, 2008).

Another gene demonstrated to be involved in GA response is the *SHI* gene, *SHORT INTERNODES*, isolated from *Arabidopsis thaliana* by Fridborg *et al.* (1999). The *shi* mutant was explained as an overexpression of the *AtSHI* gene directed by the 35S CaMV promoter inserted in the upstream region of the *AtSHI* coding region. The phenotype resembled a mutant defective in GA biosynthesis, however, the plants had elevated levels of GA, which indicated a defect in GA perception rather than biosynthesis. The plants had reduced apical dominance and an increased number of flowers, but most interestingly normal flowering time could be restored by GA application whilst the mutants remained dwarfed (Fridborg *et al.*, 1999). The separation of flowering time and elongation growth makes the *AtSHI* gene an obvious candidate for a biotechnological approach to retard ornamental plants to produce compact and bushy cultivars in horticulture.

It is now clear that *AtSHI* is a member of a family of putative transcription factors comprising nine members all expressed in *Arabidopsis* (Fridborg *et al.*, 2001; Kuusk *et al.*, 2006). The proteins are partially redundant, e.g. they can compensate for each other and they probably function as multimers (Kuusk *et al.*, 2006) to maintain auxin homeostasis (Sohlberg *et al.*, 2006; Staldal *et al.*, 2008).

Kalanchoë is a popular ornamental marketed worldwide both as indoor potted plants and as a garden plant. With an annual production of 42 million plants, *Kalanchoë* is the most produced ornamental plant in Danmark followed by Rosa hybrids (34 millions) and *Campanula* (18.5 millions), respectively (Floradania Marketing A⁄S, 2008). A compact plant growth habit is crucial for the quality of *Kalanchoë*, however, the natural growth pattern is very elongated. The *Arabidopsis SHI* (*AtSHI*) gene was chosen as candidate gene for controlling growth of *Kalanchoë* because its overexpression in *Arabidopsis* shows an attractive phenotype. Secondly, proof of transferability of the *AtSHI* phenotype among plant species would be valuable in horticulture for controlling plant growth. The *AtSHI* cDNA under control of the 35S CaMV promoter was transformed into eight *Kalanchoë* cultivars each having distinct growth habits. Various quality parameters were

envisaged and showed that overexpression of *AtSHI* resulted in more compact plants with reduced plant height and diameter. Moreover, the vegetative height of the plants was increased leading to more compact inflorescences. This is to our knowledge the first example of exploring a transgenic approach using *Agrobacterium tumefaciens*-mediated transformation to control plant compactness while still maintaining flowering time.

Results and discussion

Regeneration of transgenic SHI-lines

The *AtSHI* gene was subcloned and inserted into an overexpression vector and introduced into *Kalanchoë* by *Agrobacterium tumefaciens*-mediated transformation. An *Agrobacterium tumefaciens*-mediated transformation platform applicable for a range of *Kalanchoë* cultivars was established. It was found that the combination of 1 mg/L thidiazuron (TDZ) and subsequent addition of 0.85 mg/L gibberellin (GA_3) to stimulate formation of shoots followed by 1 mg/L of the auxin naphthalene acetic acid NAA to initiate roots, proved successful for each of the nine cultivars: *Kalanchoë blossfeldiana* 'Molly' and the interspecific hybrids '57', 'Jackie', 'Jodie', 'Naomi', 'Sarah', 'Simone', 'Suzanne' and 'Yellow African' (data not shown). However, the cultivars responded very differently to the *Agrobacterium* mediated transformation and based on the amount of callus produced, the lines could tentatively be divided into three transformation frequencies: high ('Jodie', 'Molly', 'Simone' and 'Suzanne'), medium ('57' and 'Jackie') and low ('Naomi', 'Sarah' and 'Yellow African') (data not shown). Individual lines, called SHI-lines, were selected based on their capability to grow on kanamycin containing media (100 mg L^{-1}) for several subsequent generations. Two to three lines were selected from approximately ten individual SHI-lines for each of the cultivars for further studies. One transgenic line from 'Yellow African': Yellow African-SHI-C was selected and only used in the gibberellin experiment mentioned below, as it was found to be delayed in flowering.

Presence, copy number and relative expression of *AtSHI*

The presence of the *AtSHI* gene was verified by PCR with *AtSHI* specific primers; these primers do not recognize any endogenous *Kalanchoë SHI* genes (Figure 1). Total 21 transgenic lines representing two or three lines of each of the eight cultivars were analysed for gene-copy number by quantitative real-time PCR according to the method described by Bartlett *et al.* (2008). The relative copy number of *AtSHI* inserted in the genome was calculated by standardizing the endogenous presence to glyceraldehyde phosphate dehydrogenase (GAPDH). The lines were categorized into the following significantly different groups: single copy (defined as a fold change below 2), low copy number (defined as 2–10 fold change), high copy number (fold change higher than ten) (Figure 2a). All cultivars tentatively showed at least one line with single copy insertions: 57-SHI-2, Jackie-SHI-4, Jodie-SHI-5, Jodie-SHI-8, Molly-SHI-5, Naomi-SHI-40, Sarah-SHI-1, Simone-SHI-9 and Suzanne-SHI-2. The following lines tentatively had few copies: 57-SHI-1, Jackie-SHI-5, Jackie-SHI-8, Molly-SHI-2, Naomi-SHI-2, Simone-SHI-2 and Simone-SHI-8. Finally, the following lines were tentatively categorized as having many copies: Molly-SHI-4, Naomi-SHI-1, Sarah-SHI-2, Sarah-SHI-3 and Suzanne-SHI-5 (Figure 2a).

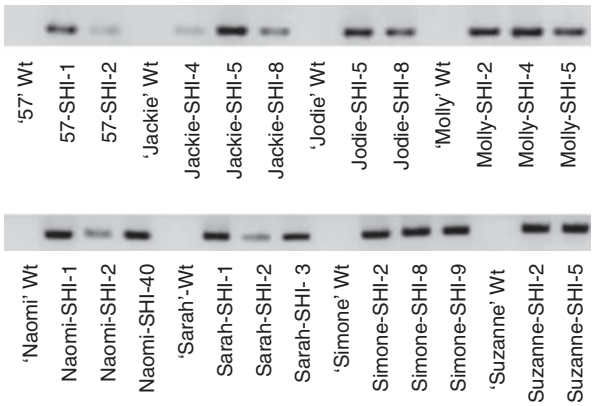

Figure 1 Characterization of transgenic *Kalanchoë*. PCR on DNA isolated from transgenic *Kalanchoë* lines using *Arabidopsis SHI* gene specific primers. DNA isolated from untransformed cultivars (Wt) served as controls. The reactions were analysed by agarose gel electrophoresis and the ethidium bromide stained PCR product of 397 bp are shown.

To determine the *AtSHI* expression levels in the transgenic *Kalanchoë* lines, relative expression of *AtSHI* was investigated by quantitative real-time RT-PCR by correlating transcript levels to endogenous GAPDH transcript levels and then comparing these values between SHI-lines and wild type (Wt) plants. *AtSHI* expression was found in all the SHI-lines (Figure 2b). The highest relative expression levels were found in 57-SHI-1, Molly-SHI-4, Naomi-SHI-1, Sarah SHI-2, Sarah SHI-3 and Susanne-SHI-5 (Figure 2b). Intermediary relative expression levels were found in Jackie-SHI-5, Jackie-SHI-8, Jodie-SHI-5, Molly-SHI-2, Molly-SHI-5, Sarah-SHI-1, Simone-SHI-2 and Simone-SHI-8,

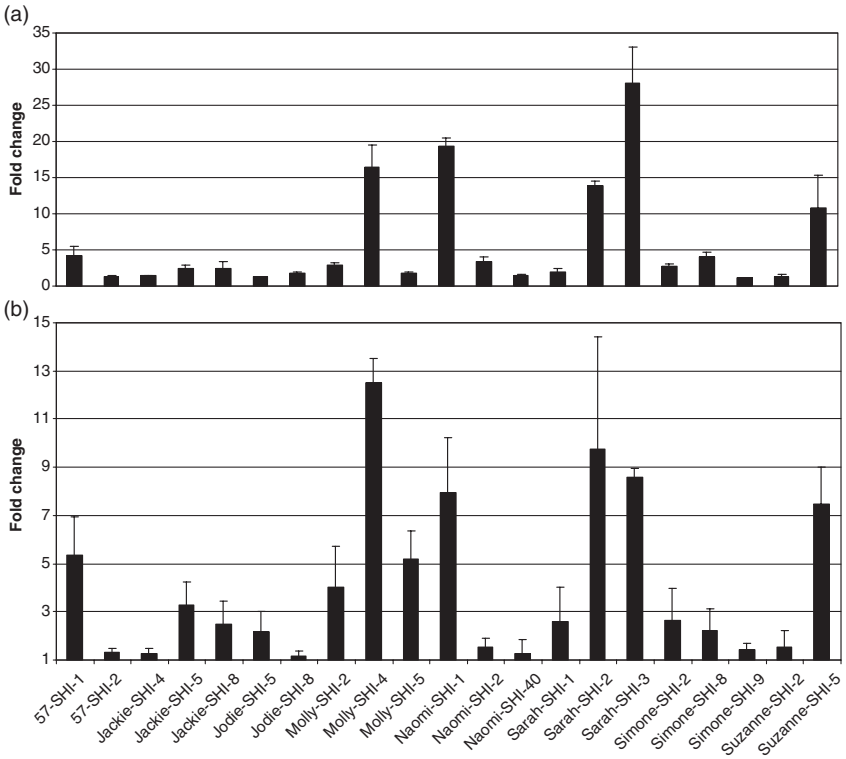

Figure 2 *AtSHI* copy numbers and *AtSHI* expression *level.* The relative presence (copy number) (a) and relative expression (b) of *AtSHI* in the transgenic lines calculated as fold change $2^{-\Delta(\Delta Ct)}$ for *AtSHI* standardized to the *GAPDH* threshold cycles (ΔCt) with the differences between the SHI-lines and Wt calculated as Δ(ΔCt). The relative presence value of *AtSHI* for each respective Wt is 0. The relative expression value for each respective Wt is 1. The data are from two independent experiments each with $n = 3$, ± standard deviation.

whereas the relative expression was low in the remaining seven lines (Figure 2b).

Plant phenotypes

In all eight transgenic *Kalanchoë* cultivars overexpression of *AtSHI* resulted in dwarfed, compact plants, however, the SHI-lines showed a range of dwarfing phenotypes, and some cultivars were more susceptible to the growth reducing effect of *AtSHI* than others, as shown in Figure 3. Data for cultivars 'Molly' and 'Suzanne' are shown in the Supporting Information. The most pronounced dwarfing effect of *AtSHI* was found in the cultivars '57' and 'Sarah' (Figure 3a,e), moreover, the individual SHI-lines within these cultivars were very similar in phenotypes. In cultivar '57,' both of the transgenic SHI-lines were one-third of the Wt height and in 'Sarah' all three SHI-lines were approximately half the height of the Wt (Figure 3a,e). Furthermore, the plant diameter of these SHI-lines was reduced to nearly half of the Wt. Overexpression of *AtSHI* in 'Naomi' and 'Suzanne' also resulted in dwarfed plants, where the lines Naomi-SHI-40, Naomi-SHI-1 and Suzanne-SHI-5 were found to be most compact (Figures 3d and S1). The plant diameter of these SHI-lines was also significantly reduced. Overexpression of *AtSHI* in the other cultivars 'Jackie', 'Jodie', 'Molly' and 'Simone' also resulted in pronounced dwarfed lines like Jackie-SHI-5, Jodie-SHI-8, Molly-SHI-2 and Simone-SHI-8 (Figures 3b, c, f and S1).

In general, all the SHI-lines showed reductions in both number of side-branches and inflorescences (Figures 3 and S1). This is in contrast to the *shi* phenotype in *Arabidopsis* (Fridborg *et al.*, 1999), which is more branched and has increased number of flowers. However, with the exception of cultivar '57', all the SHI-lines showed an increase in vegetative plant height (height of plant to the first inflorescence branch) (Figure 3). This, combined with the fact that total height is significantly reduced compared with the Wt, documents that the generative part of the plant, the inflorescence, was most severely affected by the *AtSHI* overexpression.

Interestingly, some of the most growth reduced lines like 57-SHI-1, Naomi-SHI-1, Sarah-SHI-2 and Sarah-SHI-3 (Figure 3) were also the lines with the highest amount of insert copies (Figure 2a) and the highest relative expression (Figure 2b). On the other hand, the severely growth inhib-

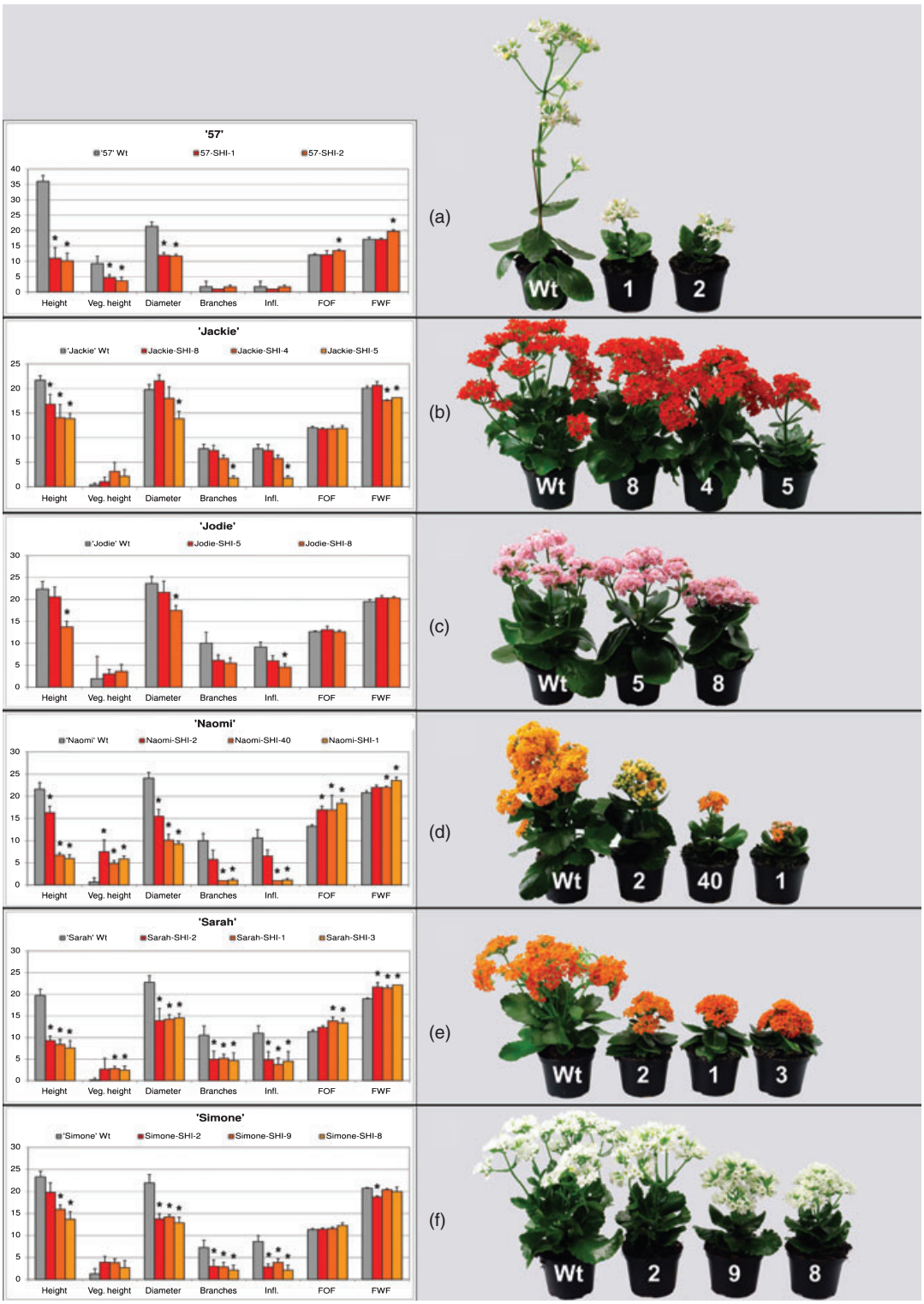

Figure 3 *Kalanchoë* SHI-lines compared with their respective Wt plants. The biometric data for plant height, vegetative plant height and plant diameter is shown in cm, number of branches (first order) and inflorescences are counted numbers, while first open flower (FOF) and first wilted flower (FWF) are counted weeks. Plants were grown in randomized plots of eight plants and the following lines were set up in three plots of eight plants: 'Jodie' Wt, Jodie-SHI-5, Jodie-SHI-8, 'Naomi' Wt, Naomi-SHI-2, 'Sarah' Wt, Sarah-SHI-2, 'Simone' Wt and Simone-SHI-2. The remaining SHI-lines were grown in one plot of eight plants. Values are shown with standard deviations and asterisks indicate values significantly different from Wt.

ited lines 57-SHI-2 and Sarah-SHI-1 showed low copy number and low relative expression.

Unlike the *Arabidopsis shi* mutant (Fridborg *et al.*, 1999), the majority of the *Kalanchoë* SHI-lines did not show significant delay in flowering. Significant delay in flowering was only observed in the SHI-lines of 'Naomi' and to a lesser extend 'Sarah' (Figure 3d,e). Although these two cultivars seemed to show retarded phenotypes leading to a delay in the appearance of the first open flower in these two cultivars (compare height and weeks to open flower in Figure 3), this correlation was not statistically significant by students *t*-test.

The duration of the flowering period was monitored and the end of flowering was determined by appearance of the first wilted flower. Most of the SHI-lines did not differ significantly from the Wt, however, the line 57-SHI-2 exhibited a significantly longer flowering period compared with both '57' Wt and 57-SHI-1 (Figure 3a). On the other hand, all Jackie SHI-lines, Simone-SHI-2, Suzanne-SHI-2 and Suzanne-SHI-5 had a significantly shorter flowering period than their corresponding Wt (Figure 3b, f and S1).

The delay in flowering was investigated further in Sarah-SHI-2 and 'Sarah' Wt, Jackie-SHI-8 and 'Jackie' Wt together with Yellow African-SHI-C and 'Yellow African' Wt by testing if gibberellin [0.1% (w/v) GA_3] applied by spraying could restore the time of flowering. These data indicated that application of GA resulted in 1 week earlier flowering in Sarah-SHI-2 and approximately 3 weeks earlier flowering in Yellow African-SHI-C (Table 1). However, application of GA to Jackie-SHI-8, which did not show any delay in flowering, did not result in an even earlier time of flowering (Table 1).

As the dwarfing effect of *AtSHI* was very pronounced in 'Sarah', this cultivar and its SHI-lines were selected for further analysis. The length of the individual branches within the inflorescences was measured and it was found that the length in the SHI-lines decreased to 12%–25% of 'Sarah' Wt branches (Table 2). The reduced length of the inflorescence branches contributed to a denser inflorescence, a parameter of crucial importance for *Kalanchoë* quality. Transcript levels of *AtSHI* were investigated by RT-PCR in vegetative stems of all 'Sarah' SHI-lines to determine if elevated transcript levels could cause the decrease in the length of the side-branches, however, transcript levels were found to be similar to the general relative expression (Figure 2) (data not shown).

Table 2 Average length of inflorescence branches (IB) in the cultivar 'Sarah'

Line	Wt	SHI-1	SHI-2	SHI-3
IB (cm)	9.9 ± 2.1	1.2 ± 0.5*	2.4 ± 0.8*	1.7 ± 0.8*
n	24	8	24	8

Values are shown in cm with standard deviations. *n* corresponds to the number of plants used in the experiment.
*Values significantly different from Wt.

The overall dwarfing effect of *AtSHI* was monitored through vegetative propagation in successive generations and found to be stable in more than five generations (data not shown). Furthermore, *Kalanchoë* SHI-lines were crossed with other cultivars and the offspring stably inherited *AtSHI* (data not shown).

Fridborg *et al.* (1999) demonstrated that overexpression of *AtSHI* led to darker green leaves in *Arabidopsis*. The *Kalanchoë* SHI-lines were measured by a portable fluorimeter on ten leaves that had been dark-adapted for 25 min by use of quantum yield of PSII photochemistry (ϕPSII) calculated as F_v/F_m (see experimental procedures for more details). A ϕPSII value of 0.832 ± 0.011 was obtained for Sarah-SHI-2 and was not different from the 0.832 ± 0.004 measured on 'Sarah' Wt leaves. Other lines were also measured and did also not reveal any differences between the SHI-lines and the respective Wt (data not shown), and no difference in leaf colour was found when plants were evaluated visually. Furthermore, we did not observe any alterations of leaf morphology of *AtSHI* expressing *Kalanchoë* (data not shown). This is in contrast to the data by Fridborg *et al.* (1999) showing that overexpression of *AtSHI* resulted in narrow leaves in *Arabidopsis*.

Table 1 Effect on flowering by applying the gibberellin GA_3

Line	'Sarah' Wt	Sarah-SHI-2	'Jackie' Wt	Jackie-SHI-8	'Yellow African' Wt	Yellow African SHI-C
–GA (weeks)	12.4 ± 0.2	13.3 ± 0.2	12.9 ± 0.3	12.3 ± 0.4	11.7 ± 0.4	14.5 ± 0.5
+GA (weeks)	11.9 ± 0.8	12.4 ± 0.2*	12.6 ± 0.7	12.0 ± 0.4	11.7 ± 0.5	11.9 ± 0.3*

Values are shown in weeks until first open flower with standard deviations. –GA plants were not spayed with gibberellin whereas +GA indicates plants spayed with 0.1% (w/v) GA_3 once a week. See Experimental procedures for more details.
*Values significantly different from controls.

Effect of *Arabidopsis SHI* promoter

The effect of the *Arabidopsis* SHI promoter was studied in the cultivar 'Molly' as constitutive expression of *SHI* showed the least effect in this cultivar (see Figure S1). Although most of the plant quality parameters investigated did not differ from the Molly SHI-lines controlled by the 35S promoter, it was found that the Molly-pSHI-4 line flowered earlier than both Wt and the SHI-lines and had the same flowering period (Table 3).

SHI genes in *Kalanchoë*

Sequences homologous to known *SHI* genes seem to be present in all vascular plants. To investigate the degree of conservation of these genes between *Arabidopsis* and *Kalanchoë*, we isolated cDNA fragments of expressed *SHI* genes by RT-PCR on isolated RNA from the *Kalanchoë* cultivar 'Celine' using a degenerate primer set. Two different fragments were isolated, both were partial sequences, but contained both the zinc finger motif and the IGGH domain specific for SHI family proteins (Figure 4a). The two sequences were 671 bp and 633 bp and were named *KbSRS1* and *KbSRS2*, respectively, for *K. blossfeldiana SHI RELATED SEQUENCES*. The GenBank accession numbers are GQ847757 and GQ847758 for *KbSRS1* and *KbSRS2,* respectively.

These two homologous sequences were 70% identical on nucleotide level and 84% on amino acid level, respectively (Figure 4a). Compared with other published sequences the nucleotide fragments showed highest similarity (68%–70% identities) to a *SHI* homologous gene from rice (Os01g0954500), and among the *Arabidopsis SHI* sequences the most similar sequence (66% identity) was the *AtLRP1.1* (At5G12330). Surprisingly, further RT-PCR experiments with species specific primers, based on the two *KbSRS* sequences, did not result in isolation of any novel *SHI* homologues, although the two *KbSRS* sequences were found several times in individual samples.

Southern blot analysis was performed with a probe covering the entire *KbSRS1* sequence on Wt 'Molly' *Kalanchoë* DNA. Hybridization was performed under low stringency conditions (61 °C) to allow recognition of related genes in the genome. This resulted in four hybridizing bands in the *Eco*RI digest and eight bands in the *Hin*dIII digest (Figure 4b). This is in agreement with the probe having an internal *Hin*dIII site and the fact that 'Molly' is a tetraploid cultivar. Some hybridizing bands appeared stronger than others, separating the bands in two groups with different similarity to the cloned gene fragment used as probe. Therefore, the Southern blot analysis further supports the hypothesis of two *SHI* genes being present in *Kalanchoë*.

These findings allow us to suggest that the *SHI* family in *Kalanchoë* is not as large as in *Arabidopsis*, but may consist of only two members. Compared with the *Arabidopsis SHI* family, the two *KbSRS* genes show highest similarity to *AtLRP1.1* (66%), but whether the expression of these genes resemble that of *AtLRP1.1* remains to be investigated.

The joint expression profiles of *KbSRS1* and *KbSRS2* were investigated in the cultivar 'Celine' using primers that amplify both sequences, but not *AtSHI*. These data indicated strongest expression in nodes, internodes, buds, young leaves and roots (Figure 4c). This expression profile was similar to the one described for *AtSHI* in *Arabidopsis* where *AtSHI* was strongly expressed in roots, stems and cauline leaves (Fridborg *et al.*, 1999). The joint expression profiles for *KbSRS1* and *KbSRS2* were also investigated in 'Sarah' SHI-lines, which showed a slightly lower expression level in Sarah-SHI-1 and a slightly higher expression level in Sarah-SHI-2, whereas the expression in Sarah-SHI-3 was comparable to 'Sarah' Wt (Figure 4d). Uniform 18S ribosomal bands were amplified as control of equal amounts of RNA (data not shown).

Table 3 Effect of the endogenous *SHI* promoter from *Arabidopsis* on flowering in the cultivar 'Molly'

Line	'Molly' Wt	Molly pSHI-4	Molly SHI-5
FOF (weeks)	11.2 ± 0.3	10.6 ± 0.2*	11.1 ± 0.2
FWF (weeks)	14.4 ± 0.3	14.1 ± 0.1	13.9 ± 0.0

Values are shown in weeks until first open flower (FOF) and first wilted flower (FWF). Values are shown with standard deviations, $n = 16$.
*Values significantly different from Wt.

Conclusion

The present work shows that growth retardation using the *AtSHI* gene in a range of *Kalanchoë* cultivars is a very promising alternative to chemical growth retardants. Experimental evidence for reduced height and plant diameter due to the overexpression of *AtSHI* was presented. The overall study of overexpression of *AtSHI* in *Kalanchoë* revealed some significant physiological differences compared to overexpression in *Arabidopsis*. Most importantly, the majority of the SHI-lines were not delayed in flower-

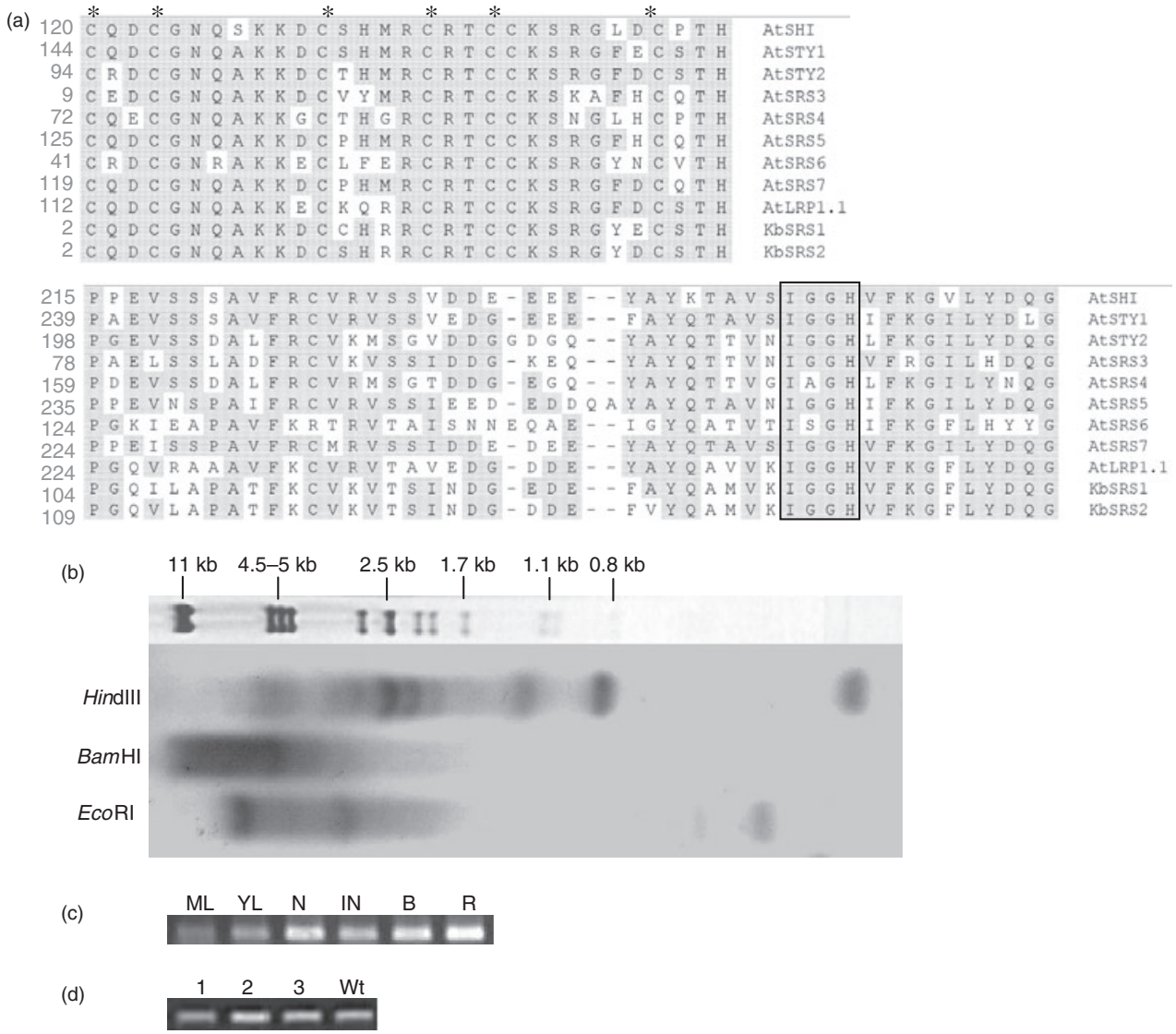

Figure 4 Characterization of *Kalanchoë SHI* genes. (a) Alignments of the deduced amino acid sequences of two conserved SHI protein domains from the nine *SHI* genes from *Arabidopsis* and the two homologous genes found in *Kalanchoë* 'Celine'. The zinc finger motif starting with residue number 120 in AtSHI is shown above with asterisks marking the conserved residues as assigned by Fridborg *et al.* (2001). The IGGH domain is boxed in the alignment starting with residue number 215 in AtSHI. (b) Southern blot analysis of Wt *Kalanchoë* 'Celine' DNA using the *KbSRS1*fragment as a probe. DNA (10 μg) were digested with single enzymes *Hin*dIII, *Bam*HI and *Eco*RI as indicated. (c) Joint expression pattern of *KbSRS1* and *KbSRS2* shown by RT-PCR on 40 ng of DNase treated total RNA from various tissues of *Kalanchoë* 'Celine'. ML, mature leaves; YL, young leaves; N, nodes; IN, internodes; B, buds; R, roots. (d) Joint expression pattern of *KbSRS1* and *KbSRS2* shown by RT-PCR on 40 ng of DNase treated total RNA from the 'Sarah' lines as indicated by the numbers. See Experimental procedure for more details.

ing, this feature is very important in the ornamental plant industry, as delayed flowering increases the production costs significantly. Our results also showed that exogenously application of gibberellic acid in low concentrations can restore the period of flowering in SHI-lines that were otherwise delayed. Furthermore, in contrast to the effect of overexpression of *AtSHI* in *Arabidopsis*, no morphological changes in leaf shape were observed in *Kalanchoë*.

Significant differences in phenotypes were found for some of the individual SHI-lines within a cultivar. This feature can be utilized in the ornamental production of *Kalanchoë* as a plethora of desired plant products, ranging from very dwarf plants to plants without elongated inflorescences, could be produced. In addition, utilization of the native *SHI* promoter from *Arabidopsis* resulted in plants with an earlier and prolonged period of flowering. Although our data did not indicate the presence of a large

family of *SHI* genes in *Kalanchoë*, the sequences of the related genes encode proteins that are very well conserved in the protein functional domains. Considering this conservation of the SHI proteins between these two species and the dwarfing phenotypes of the transgenic plants, we suggest that the *Arabidopsis* SHI protein is functional in *Kalanchoë* and hence act in a similar way as in the *shi* mutant. This is supported by the similar expression profiles observed for *AtSHI* in *Arabidopsis* and *KbSRS1* and *KbSRS2* in *Kalanchoë*. Overall these results give hope that overexpression of *SHI* genes can be applied to several other important crops as well.

Experimental procedures

Constructs

35S::*AtSHI* construct

The open reading frame of *SHI* from *A. thaliana* ecotype 'Landsberg *erecta*' (L*er*) was available in a TOPO pCR2.1 vector (Invitrogen, Carlsbad, CA, USA) kindly provided by Eva Sundberg and Joel Sohlberg, Dept. of Plant Biology and Forest Genetics, Swedish University of Agricultural Sciences, Uppsala, Sweden. The pCR2.1 vector containing the *AtSHI* coding region was sequenced and shown to contain five nucleotide substitutions compared with the ecotype 'Colombia' gene, At5G66350. The *AtSHI* cDNA was isolated as a *Bam*HI fragment and ligated into the pRT100 vector (Töpfer *et al.*, 1987), between the 35S promoter and the polyA signal. The ligation mix was transformed into *E. coli* Top10F' competent cells (Invitrogen) and selected on LB plates supplemented with ampicillin 50 mg L^{-1} (Amp50), 0.1 mM IPTG and X-gal 40 μg mL^{-1} for blue/white screening. White colonies were grown overnight in LB Amp50 medium and plasmids were purified using CTAB precipitation (Lander *et al.*, 2002). The orientation of the *AtSHI* coding region was determined by sequencing. The cassette, 35S-*AtSHI*-polyA was transferred as a *Hin*dIII fragment to the binary vector pKanIntron described by Libiakova *et al.* (2001) giving rise to the construct pKanIntron-35S-*AtSHI*-polyA. This final construct was designated 35S-*SHI*.

pAtSHI::*AtSHI* construct

The *SHI* open reading frame from *Arabidopsis* Wt L*er* was amplified from the construct mentioned above using the following primers: forward: 5'-GGG GAC AAG TTT GTA CAA AAA AGC ATT CAT GGC AGG ATT TTT CTC GTT AGG-3', reverse: 5'-GGG GAC CAC TTT GTA CAA GAA AGC TGG GTC TCA AGA TCT TGA GTT GGA GAA G and recombined into the pK2GW7 vector (Karimi *et al.*, 2002) using the Gateway clonase II system (Invitrogen, Carlsbad, CA, USA) following manufacturer's protocol. The 35S promoter was subsequently excised from the vector using *Sac*I and *Spe*I. The endogenous *SHI* promoter was cloned from *Arabidopsis* Wt Col DNA and subsequently digested with *Sac*I and *Spe*I. Finally, the promoter was ligated into the vector. One microliter of the ligation mix was used to transform Omnimax chemically competent cells (Invitrogen, Carlsbad, CA, USA) according to producer's protocol.

Plant material

Kalanchoë cultivars '57', 'Jackie', 'Jodie', 'Molly', 'Naomi', 'Sarah', 'Simone', 'Suzanne' and 'Yellow African' (Knud Jepsen A/S, Hinnerup, Denmark) were established *in vitro* by sterilizing nodal cuttings for 15 min in 2% (v/v) NaOCl with 0.03% (v/v) Tween 20. Sterilization was performed with gentle agitation (50 rpm) before the cuttings were washed three times in sterile water. Explants were placed on standard MS medium with Gamborg vitamins (Duchefa, Haarlem, The Netherlands) (Murashige and Skoog, 1962; 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 media 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, Eindhoren, The Netherlands).

Transformation of *Kalanchoë* cultivars

The binary vectors were introduced into *Agrobacterium tumefaciens* strain C58C1/GV3850 by electroporation. Colonies were selected on YEP plates containing rifampicin 100 mg/L (Rif100) and ampicillin 50 mg/L (Amp50) for the 35S-*SHI* construct and Rif100 and spectinomycin 100 mg/L for the *pAtSHI::AtSHI* construct, respectively. The constructs were verified by plasmid purification and restriction enzyme digests and sequencing. Transformation of *Kalanchoë* cultivars was performed as described previously (Topp *et al.*, 2008) with the exception that the *Agrobacterium* was resuspended in YEP containing 15 mg/L acetosyringone for the transformation solution. After 2 days, co-cultivation on basic media containing 15 mg/L acetosyringone leaf discs were transferred to regeneration medium [basic medium supplemented with 1 mg/L thidiazuron (TDZ), 100 mg/L timentin (Tim100), 500 mg/L cefotaxime (Cef500) and 100 mg/L kanamycin (Kan100)]. All hormones and antibiotics were purchased from Duchefa (Saveen & Werner, Malmö, Sweden). Leaf discs were transferred to fresh selective regeneration media every third week. Shoots were placed in containers with basic medium, Kan100, Tim100, Cef500, Gibberellin (GA_3) 0.85 mg/L and 1 mg/L of the auxin naphthalene acetic acid (NAA) (Sigma-Aldrich, St. Louis, MO, USA) were added to stimulate formation of shoots and roots, respectively, when needed. Approximately ten independent transgenic lines were established for each parental cultivar. Rooted shoots were transferred to rock wool substrate blocks (Grodan, Hedehusene, Denmark) grown under greenhouse conditions for 2–3 months to establish mother stocks before cuttings were harvested. Cuttings from each transgenic line were rooted on soil in 10.5 cm pots at 16 h day/8 h night for 3 weeks, then placed at short day conditions, 10 h day/14 h night for flower induction. During the day period, light intensity was 150 μmol/m^2/s^2 and day length was 10 h with a light period from 7 AM to 5 PM. The temperature was 16 °C between 6 AM to 8 AM, 19 °C between 8 AM to 8 PM and 21 °C between 8 PM to 6 AM. These growth settings represent the conditions used in commercial *Kalanchoë* production. The relative humidity varied between 17% and 52%. Plants transformed with the 35S-*AtSHI* construct were grown in randomized plots of eight plants and the following lines were set up in three plots of eight plants: 'Jodie' Wt, Jodie-SHI-5, Jodie-SHI-8, 'Naomi' Wt,

Naomi-SHI-2, 'Sarah' Wt, Sarah-SHI-2, 'Simone' Wt and Simone-SHI-2. The remaining SHI-lines were grown in one plot of eight plants. Each plot was surrounded by border plants ('Simone' Wt) to ensure equal growth conditions for the plants in the plots. The *pAtSHI::AtSHI* construct was transformed into the cultivar 'Molly', and the line Molly-pSHI-4 was selected and together with 'Molly' Wt and Molly-SHI-5 grown as described above in two plots of eight plants.

Evaluation of transgenic lines

Plantlets that remained green after successive rounds of subcultivation on media containing kanamycin (Kan 100) were investigated for the presence of the *AtSHI* gene by PCR with primers SHI-s (5′-ACT CTA ACG CTG ACG GTG GA-3′) and SHIrel_as (5′-TGC TGA CCG GTA GAA AGC TG-3′). These primers are specific for the *Arabidopsis* gene and do not result in any amplification product from Wt *K. blossfeldiana* DNA. Denaturation at 95 °C for 15 min and amplification of 40 cycles (95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min.) followed by one cycle of 72 °C for 7 min was performed in a Veriti Thermal Cycler from Applied Biosystems (Foster City, CA, USA), and two-three confirmed 35S-*SHI* containing lines for each of the cultivars were selected for further analysis as mentioned above.

Quantitative real-time PCR for determining transgene copy number

Genomic DNA was isolated by optimization of a DNA extraction protocol for Agavaceae (Keb-Llanes *et al.*, 2002) as described by Christensen *et al.* (2008). Hundred nanogram of DNA was used in each 25 μL PCR reaction and three replicates were carried out for each sample. Real-time PCR on DNA was carried out as described in Bartlett *et al.* (2008) with the exception that the reactions for *AtSHI* and the reference gene *GAPDH* both individually were labelled with SYBR green by using the iQ SYBR Green Supermix (Bio-Rad, Copenhagen, Denmark) according to supplier's instructions. The final primer concentration was 200 nM for each primer, 5′-CAG CTC ACT TGA AGG GTG GT-3′ and 5′-GCC TTC GGA TTC CTC CTT GAT A-3′ was used as forward and reverse primer, respectively, for *GAPDH* whereas the above mentioned primer SHI-s and Znfing-as (5′-CAA CCT CCA ACC CTG AAG TG-3′) were used for the *AtSHI* gene. The reactions for Real-time PCR were performed on an ICycler instrument (Bio-Rad, Copenhagen, Denmark) using denaturing at 95 °C for 15 min followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C. Threshold cycles (Ct), (defined as cycle were the signal exceeds ten times the standard deviation of the baseline), for *AtSHI* were standardized to the *GAPDH* Ct (ΔCt). The relative quantification of target gene *AtSHI* between the different SHI-lines was determined as $2^{-\Delta\Delta Ct}$. PCR conditions were optimized for equal amplification efficiency between target gene *AtSHI* and housekeeping gene *GAPDH* (Livak and Schmittgen, 2001). Values are based on three replicates and repeated twice.

Relative expression of the *AtSHI* gene

Total RNA was extracted from plant tissue (leaves and stem) by using the RNeasy Plant Mini Kit (Qiagen, Copenhagen, Denmark). Manufacturer's protocol was followed except for the lysis buffer; RLC buffer was chosen and supplemented with 3% (v/v) PEG 20.000 (Fluka, Buchs, Switzerland). Total RNA, 500 ng, 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, Copenhagen, Denmark) according to manufacturer's protocol. Real-time PCR was conducted as described above and the amount of template in each reaction corresponded to 25 ng total RNA.

Expression of *KbSRS* genes

Total RNA from *Kalanchoë* was isolated from different tissues of the cultivar 'Celine' and from 'Sarah' SHI-lines and Wt. The RNA was DNase treated as described above. The platinum quantitative RT-PCR ThermoScript one step system from Invitrogen was used according to manufacturer's instructions with 40 ng RNA as template and *Kalanchoë* specific primers amplifying both *KbSRS1* and *KbSRS* Shi-Kbsp193 5′-CTT CAT CGG TGT CGA TGA GTG TG-3′ and Shi-Kbsp384 5′-TGA ACG TGG CCG GCG CCA-3′. The PCR program for the evaluation of transgenic lines described above was used using 28 cycles.

Plant quality analysis

To evaluate the effect of the presence of the *AtSHI* gene in the various *Kalanchoë* lines, a range of plant quality specific parameters were scored: Weeks until first open flower, weeks until first wilted flower, total plant height, vegetative plant height (defined as the height to the last regular leaf pair before the inflorescence), amount of side-branches of first order, amount of inflorescences (each with the presence of regular leaf pairs) and plant diameter measured at the broadest length around the plant base. For the 'Sarah' Wt and SHI-lines, the lengths of the individual branches in the inflorescences were also measured.

Gibberellin application

Sarah-SHI-2, 'Sarah' Wt, Jackie-SHI-8, 'Jackie' Wt, Yellow African-SHI-C and 'Yellow African' were grown as described above in two plots of eight plants for both treated and untreated plants and exogenously sprayed with 0.1% (w/v) gibberellin GA_3 once every week starting from the transfer to a short day regime.

Photosynthetic capacity

Quantum yield of PSII photochemistry (ϕPSII) was measured in 'Sarah' Wt and SHI-lines by a portable fluorimeter (Handy plant efficiency analyser; Hansatech Instruments Ltd., King's Lynn Norfolk, UK) on plants that had been dark-adapted for 25 min. ϕPSII was calculated as F_v/F_m, where $F_v = F_m - F_o$. The abbreviations F_m and F_o describes the fluorescence yield at fluorescence maximum and fluorescence minimum, respectively. Ten randomly selected leaves were measured for each line.

Effect of native *AtSHI* promoter

Kalanchoë 'Molly', Molly-SHI-5 and Molly-pSHI-4 were grown and analysed as described above in two plots of eight plants.

RT-PCR isolation of *Kalanchoë SHI* genes

Total RNA was isolated from stem tissue of adult greenhouse grown plants of the cultivar 'Celine' (Knud Jepsen A/S, DK) using the RNeasy Plant Mini kit and subjected to DNase treatment using Q1 DNase (Promega, Madison, WI, USA) as described above. Primers *AtSHI*780 (5′-C(A/C)A GCT GCC AGG A(C/T)T G(C/T)G G(G/C)A A-3′) and *AtSHI*1236 (5′-TCC ACC GCC CGA (G/C)GA (G/T)C(C/T) (G/T)C(A/T) C(G/T)C G(A/G)C C-3′) were designed based on alignment of *SHI* from *Arabidopsis* and a homologous genomic fragment from rice (*Oryza sativa*) from NCBI GenBank. Using the Superscript One Step RT-PCR System from Invitrogen, RT-PCR reactions were carried out according to the instructions provided by the manufacturer, and the products were cloned directly into vector pCR2.1 using the TA TOPO cloning technology (Invitrogen, Carlsbad, CA, USA). Plasmid inserts were sequenced at Eurofins MWG Operon (DE).

Southern blot probed with *KbSRS1*

Presence of endogenous *SHI* genes in *Kalanchoë* was analysed by Southern blot. The blot on *Kalanchoë* 'Molly' DNA was performed as previously described (Topp *et al.*, 2008) using a probe amplified from the *KbSRS1* pCR2.1 plasmid with primers TOPO-Forw (5′-CCA GTG TGA TGG ATA TCT GCA-3′) and TOPO-Rev (5′-ACG GCC GCC AGT GTG CT-3′) and hybridization at 61 °C.

Statistics

Data were statistically analysed used the Microsoft Excel package. Significant differences were calculated using students *t*-test. Significant differences are indicated by asterisks in the figures.

Acknowledgements

The authors would like to thank Sille Høgly Petersen and Camilla Falk for technical assistance, Else Bollerup and Kai Lønne (Knud Jepsen A/S) for growing *Kalanchoë* in experimental setup and for fruitful discussions and Louise Jepsen (Knud Jepsen A/S) for assistance with the plant photos. Part of the work described was funded under the Danish Ministry of Food, Agriculture and Fisheries (DFFE).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 'Molly' and 'Suzanne' *Kalanchoë* SHI-lines compared with their respective Wt plants.

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