

# Manipulation of *MKS1* gene expression affects *Kalanchoë blossfeldiana* and *Petunia hybrida* phenotypes

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## Summary

The establishment of alternative methods to chemical treatments for growth retardation and pathogen protection in ornamental plant production has become a major goal in recent breeding programmes. This study evaluates the effect of manipulating MAP kinase 4 nuclear substrate 1 (MKS1) expression in *Kalanchoë blossfeldiana* and *Petunia hybrida*. The *Arabidopsis thaliana* MKS1 gene was overexpressed in both species via *Agrobacterium*-mediated transformation, resulting in dwarfed phenotypes and delayed flowering in both species and increased tolerance to *Pseudomonas syringae* pv. *tomato* in transgenic *Petunia* plants. The lengths of the stems and internodes were decreased, while the number of nodes in the transgenic plants was similar to that of the control plants in both species. The transgenic *Kalanchoë* flowers had an increased anthocyanin concentration, and the length of the inflorescence stem was decreased. The morphology of transgenic *Petunia* flowers was not altered. The results of the *Pseudomonas syringae* tolerance test showed that *Petunia* plants with one copy of the transgene reacted similarly to the nontransgenic control plants; however, plants with four copies of the transgene exhibited considerably higher tolerance to bacterial attack. Transgene integration and expression was determined by Southern blot hybridization and RT-PCR analyses. MKS1 in wild-type *Petunia* plants was down-regulated through a virus-induced gene silencing (VIGS) method using tobacco rattle virus vectors. There were no significant phenotypic differences between the plants with silenced MKS1 genes and the controls. The relative concentration of the MKS1 transcript in VIGS-treated plants was estimated by quantitative RT-PCR.

**Keywords:** compact growth, MKS1, MAP kinase 4 substrate 1, growth retardants, pathogen tolerance, ornamental plants.

## Introduction

Mitogen-activated protein kinase (MAPK) cascades play an important role in plant defence responses. These cascades are activated by environmental signals, which stimulate plasma membrane receptors and trigger activation of a downstream signalling network involving MAPK cascades that control the activity and synthesis of proteins, hormones and other substances important in pathogen resistance (Morris, 2001). It has been shown that endogenous salicylic acid (SA) accumulation induces the translocation of the systemic acquired resistance (SAR) signal (Loake and Grant, 2007). SAR is a form of broad-spectrum pathogen resistance in plants that is activated quickly and lasts for up to a few months (Fu and Dong, 2013; Ross, 1961). Within hours, the induced signal spreads from the infected tissue to the uninfected systemic tissue (Shah and Zeier, 2013). SAR activation is indicated by the increased expression of pathogenesis-related (PR) genes (Malamy *et al.*, 1990). PR proteins encoded by PR genes are induced through the action of signalling compounds such as salicylic acid, jasmonic acid or ethylene. PR proteins have antimicrobial properties that function via contact toxicity and hydrolytic effects on cell walls and may be involved in defence signalling (van Loon *et al.*, 2006).

It has been shown that SA analogues, such as 2,6-dichloroisonicotinic acid (INA) and benzothiadiazole S-methyl ester (BTH), induce the expression of the same group of PR genes (Friedrich

*et al.*, 1996; Görlach *et al.*, 1996; Lawton *et al.*, 1996; Métraux *et al.*, 1991). Petersen *et al.* (2000) reported that MAP kinase 4 (MPK4) negatively regulates SAR defence responses in *Arabidopsis thaliana* (At). MPK4 forms a complex with MKS1 (MAP Kinase 4 Substrate 1) and the WRKY33 transcription factor. Upon infection, MPK4 phosphorylates MKS1 and releases WRKY33 and MKS1. WRKY33 regulates the expression of PAD3 (phytoalexin-deficient 3), which leads to the synthesis of the antimicrobial substance camalexin (Mao *et al.*, 2011; Qiu *et al.*, 2008). Andreasson *et al.* (2005) showed that MKS1 overexpression in wild-type *Arabidopsis* activated salicylic acid (SA) resistance but did not interfere with the induction of defence genes by jasmonic acid. Plants exhibited semi-dwarfed phenotypes, elevated levels of pathogenesis-related protein 1 (PR1), an almost fourfold increase in SA levels and showed increased resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) (Andreasson *et al.*, 2005; Petersen *et al.*, 2010). SA, chemically known as 2-hydroxy benzoic acid, is a phenolic compound that is synthesized by plants and consists of an aromatic ring with a hydroxyl group or its functional derivative. In addition to the induction of SAR, SA also plays a role in plant growth, flower induction, the uptake of ions and thermogenesis. SA affects stomatal movement and ethylene biosynthesis, enhances the level of photosynthetic pigments and the photosynthetic rate and also modifies the activity of some important enzymes (Raskin, 1992; Vlot *et al.*, 2009). Several studies, mostly in *Arabidopsis*, have shown that plants with increased SA levels exhibit compact phenotypes

(Bowling *et al.*, 1994; Lee *et al.*, 2006; Li *et al.*, 2001; Petersen *et al.*, 2000).

*Kalanchoë blossfeldiana* and *Petunia hybrida* are economically important ornamental plant species. Approximately 77 million *Kalanchoë* and 14 million *Petunia* plants are sold per year (Key Figures 2012, Flora Holland, 2013). *Kalanchoë* and *Petunia* differ with respect to their growth habit: *Kalanchoë* is an indoor succulent plant, whereas *Petunia* is an outdoor herbaceous plant. Both species also differ in the photoperiodic induction of flowering. *Kalanchoë* flowering is induced under short-day (SD) conditions, whereas *Petunia* flowering is induced under long-day (LD) conditions. Both species have been studied with the aim of determining how to improve various qualities. To delay petal senescence, both species were transformed with the ethylene resistance *etr1-1* gene under the control of the flower-specific promoter (*fbp1*) (Bovy *et al.*, 1999; Sanikhani *et al.*, 2008). These species were also genetically modified to alter their petal colour (Meyer *et al.*, 1987; Nielsen *et al.*, 2005; Oud *et al.*, 1995). *Kalanchoë* cultivars have been the subject of several independent studies aiming to reduce the growth of the vegetative and generative stem, including constitutive overexpression of gibberellin 2 oxidase (*GA<sub>2</sub>ox*) (Gargul *et al.*, 2013), transformation with *Agrobacterium rhizogenes* (Christiansen *et al.*, 2008), silencing of gibberellin 20 oxidase (*GA<sub>20</sub>ox*) under an ethanol-inducible promoter (Topp Hovbye *et al.*, 2008) and overexpression of the short internodes gene (*SH1*) under the control of the 35S and *SH1* promoters (Lütken *et al.*, 2010). Growth retardation in *Kalanchoë* is likely to be more obvious due to its vegetative and generative growth habit. During flower induction, *Kalanchoë* produces an elongated inflorescence stem, which decreases the ornamental value of the potted plant. Therefore, during commercial production, the plants are treated with chemical growth retardants. The multiple applications of chemicals depend on the stage of development and the specific *Kalanchoë* cultivar, as was previously described by Gargul *et al.* (2013). Increased tolerance to pathogens would be an additional advantage. The phenotypic appearance and resistance to biotic and abiotic stresses are usually maintained by chemical treatments applied during commercial plant production. Reducing the number of chemical treatments, either growth retardants or crop protection chemicals, would undoubtedly be beneficial to the environment and would substantially decrease the costs of the production process (Daughtrey and Benson, 2005). SAR-inducing chemicals, such as acibenzolar-S-methyl (ASM), are available. ASM was tested on different ornamental plants; however, the effects of application have been inconsistent depending on the plant species. For

example, ASM application completely suppressed a *Phytophthora infestans* infection in *Petunia*, while the same treatment did not yield positive results in *Solanum lycopersicum* (Becktell, 2005).

To our knowledge, studies aiming to understand the influence of MKS1 on the size and pathogen immunity of the plants have not been conducted on any ornamental species. The present study focused on investigating the phenotypic changes caused by constitutive (CaMV35S) overexpression of *Arabidopsis* MKS1 in *Kalanchoë* and *Petunia*. Transgenic *Petunia* plants were tested for tolerance to *Pseudomonas syringae* pv. *tomato*. In addition, MKS1 was down-regulated in *Petunia* using VIGS.

## Results and discussion

### RT-PCR and Southern blot hybridization of transgenic plants

All of the investigated *Kalanchoë* and *Petunia* lines showed positive amplification by RT-PCR with primers targeted against the *AtMKS1* transgene and the housekeeping genes *KbPP2* (for *Kalanchoë*) and *CYP* (for *Petunia*) (Table 1, Figure 1).

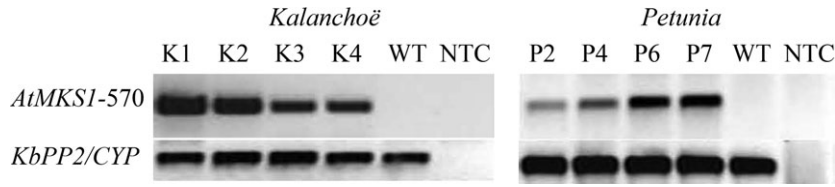
Southern blot hybridization with an *AtMKS1* probe revealed the integration of four copies of the transgene into *Kalanchoë* clone K1, two copies into clone K2 and one copy each into clones K3 and K4 (Figure 2a). *Petunia* clones P2 and P4 contained one copy of the T-DNA, and clones P6 and P7 contained four copies of the T-DNA in the genome (Figure 2b).

### Phenotypic evaluation of the vegetative growth of transgenic lines

All transgenic lines of both species exhibited significant reductions in height and internode length compared with control plants. However, the number of nodes in the transgenic plants was similar to that of the control plants. After 5 weeks of observations, the length of the stem of the transgenic *Kalanchoë* lines varied between 5.5 and 8.5 cm, while that of the control plants varied between 14.5 and 18.5 cm (Figures 3a and 4a). The stem length of the nontransgenic *Petunia* plants was 16–22 cm, while the stems of the transgenic *Petunia* lines were 8–12 cm long (Figures 3c and 5a). After 5 weeks, the number of nodes increased from 6 to 8 in the control and transgenic *Kalanchoë* plants, from 13 to 20 in the *Petunia* control plants and from 13 to 22 in the *Petunia* transgenic lines (Figures 4c and 5c). For both species, the internode length of the transgenic lines was two times shorter than that of the nontransgenic control plants on average (Figures 4b and 5b). The results of the present study correspond to the results in *Arabidopsis*, because the height of

**Table 1** Primer names, sequences and amplicon characteristics

Primer name	Target sequence	Directionality	Sequence (5'–3')	Amplicon size (bp)
<i>AtMKS1</i> -570	<i>AtMKS1</i> cDNA in RT-PCR	Forward	CCAAAGACAACCTGCAAACCA	570
		Reverse	TGCTACCAAATCCAATCAA	
<i>PhVIGS</i> -134	<i>MKS1</i> in <i>Petunia</i> cDNA	Forward	CCACTTCAGCAACTGCCTCGT	134
		Reverse	TCCTTCAGGGTTCTTGTTTTCTC	
<i>PhVIGS</i> -264	<i>MKS1</i> in <i>Petunia</i> cDNA	Forward	CGGAAAGTCACCGAGAAGAG	264
		Reverse	GCAGTTGCTGAAGTGGAACA	
<i>KbPP2</i>	<i>Kalanchoë</i> protein phosphatase 2 gene	Forward	GGGGAAGTTTGCTGCTACTG	255
		Reverse	GCAACCATGTAACGAACACG	
<i>CYP</i>	<i>Petunia</i> cyclophilin gene	Forward	AGGCTCATCATCCACCGTGT	111
		Reverse	TCATCTGCGAAGCTAGCACCG	



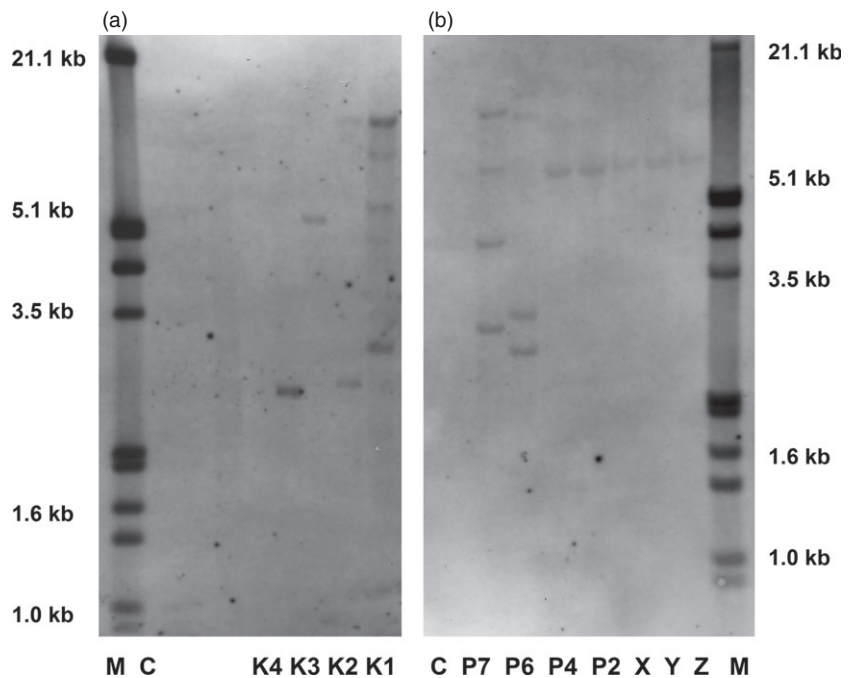
**Figure 1** RT-PCR of transgenic *Kalanchoë* and *Petunia* lines. WT indicates wild-type nontransgenic control cDNA, and NTC is a no-template control. RT-PCR was performed using the *AtMKS1-570* primer pair for both species. The *KbPP2* primer pair was used for *Kalanchoë*, and the *CYP* primer pair was used for *Petunia* (Table 1).

both species was significantly reduced. The decreased growth of the transgenic lines may be related to the higher SA levels. RT-PCR analysis demonstrated that *AtMKS1* is expressed in the investigated transgenic lines (Figure 1). Andreasson *et al.* (2005) revealed a correlation between the overexpression of *MKS1* and the SA concentration. 35S-*MKS1* transgenic plants contained approximately 13 500 ng of SA per g fresh weight of leaf tissue, whereas wild-type (WT) plants contained approximately 3500 ng of SA per g fresh weight of leaf tissue. Several studies revealed that a constitutive increase in endogenous SA might negatively affect cell size and endo-reduplication ability, leading to a dwarf-like phenotype. This phenomenon has been described in *cpr1* (constitutive expression of *PR* gene 1; Bowling *et al.*, 1994), *cpr5* (constitutive expression of *PR* gene 5; Bowling *et al.*, 1997), *acd6-1* (accelerated cell death; Rate *et al.*, 1999) and *agd2* (aberrant growth and death; Rate and Greenberg, 2001) *Arabidopsis* mutants. On the contrary, plants expressing high levels of the *nahG* bacterial gene, which encodes salicylate hydroxylase (the enzyme that converts SA to catechol), accumulate very low levels of SA, fail to express *PR* genes and are defective in SAR (Delaney *et al.*, 1994; Gaffney *et al.*, 1993). These plants have a higher growth rate (Abreu and Munné-Bosch, 2009; Du *et al.*, 2009). Nevertheless, Vanacker *et al.* (2001) showed that SA can influence cell enlargement and cytokinesis in a positive or negative way. The influence of SA on cell growth and division is very

complex and depends on the circumstances in which signalling takes place. In *Arabidopsis cpr5* and *mpk4* mutants, which accumulate higher SA concentrations, the expression levels of the xyloglucan endotransglucosylase/hydrolase genes *XTH8*, *XHT17* and *XTH31* were considerably down-regulated; however, there was no difference in the expression levels of these genes in *nahG* plants (Miura *et al.*, 2010). Xyloglucan endotransglucosylase/hydrolase genes encode enzymes that are involved in cell wall loosening and expansion (Rose *et al.*, 2002). Therefore, higher SA levels might lead to a smaller cell size, which might contribute to the dwarf-like phenotypes in these plants.

**Phenotypic evaluation of reproductive growth of transgenic lines**

Flowering in the transgenic lines of both species was delayed compared with that in nontransgenic control plants. Considering their commercial production, delayed flowering presents a significant disadvantage for ornamental plants. An extended duration of flower induction results in a delayed introduction to the market, which influences the costs of plant production. Contrasting results have been observed in *Kalanchoë* species overexpressing the *AtSH1* gene, which exhibited compact phenotypes but showed no effect on flowering time (Lütken *et al.*, 2010). In the present study, the first open flower of transgenic *Kalanchoë* lines appeared 15–20 days later than that of the



**Figure 2** Southern blot autoradiogram of transgenic *Kalanchoë* (a) and *Petunia* (b) plants. Genomic DNA was digested with *Bam*HI (*Kalanchoë*) and *Hind*III (*Petunia*) and analysed using a DIG-labelled probe formed using the *AtMKS1-570* primer pair (Table 1). M - DIG-labelled DNA molecular weight marker III; C - nontransgenic control; X, Y, Z - transgenic *Petunia* plants not described in this paper.

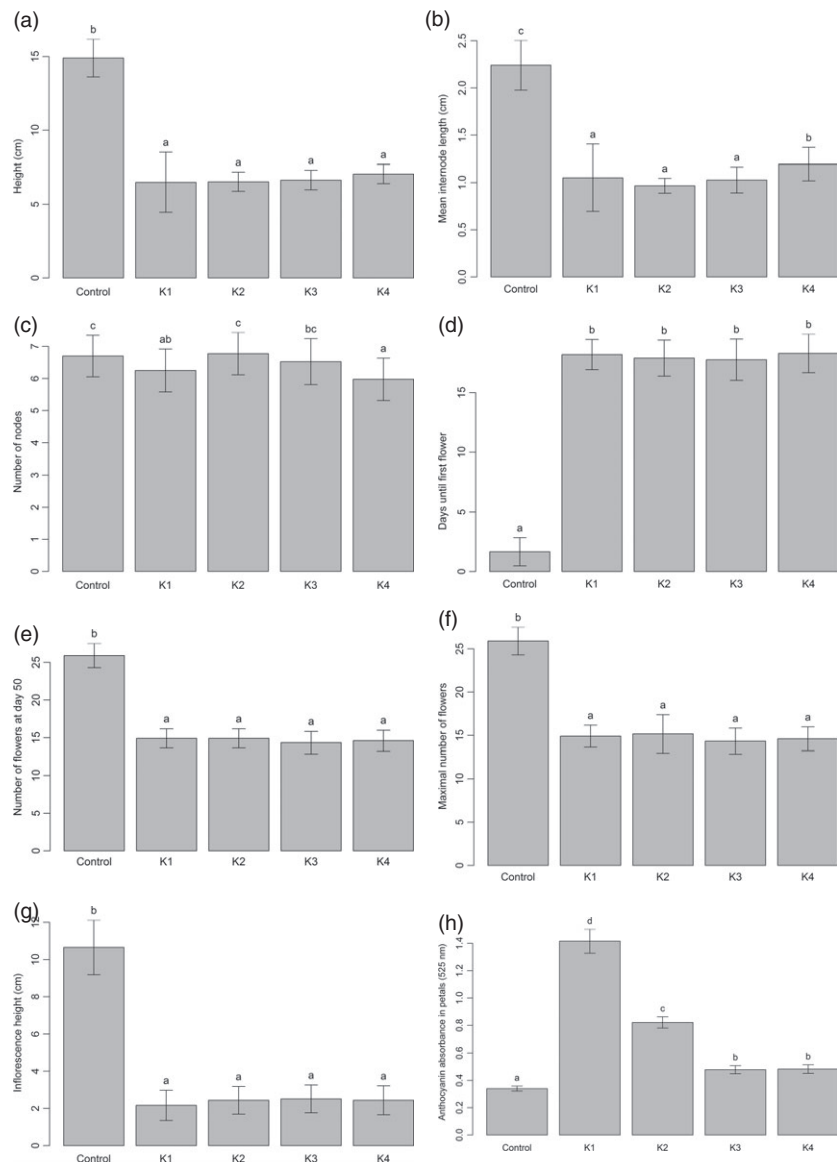


**Figure 3** Control and transgenic lines grown under greenhouse conditions. (a) *Kalanchoë* plants (C – control); (b) flowers of the K1 line; (c) regenerated *Petunia* control and transgenic line P6 with the same number of nodes.

controls, while *Petunia* transgenic lines developed their first open flower between 6 and 11 days after the first open flower was observed in control plants (Figures 4d and 5d). Flowering observations were recorded daily for 50 days. At day 50, the transgenic *Kalanchoë* lines had between 11 and 18 flowers per inflorescence, while the nontransgenic plants had 23–30 flowers per inflorescence (Figure 4e). Additionally, the number of flowers was still increasing for both transgenic and nontransgenic lines at day 50 (data not shown). *Petunia* transgenic lines P2 and P4 reached the maximal number of open flowers (13 per plant) at day 37, while the control plants reached a maximum of 15 open flowers per plant at the same time (Figure 5f). Transgenic lines P6 and P7 reached the maximum number of open flowers on day 48, and in most of the plants from these two lines, this number continued to increase over time (Figure 5e,f). The inflorescence stems of all transgenic *Kalanchoë* lines were almost five times shorter than those in control plants at 5 weeks after the opening of the first flower (Figure 4g). In contrast to the results presented here, other studies have shown that SA is a positive regulator of the flower induction process in plants. This positive regulation usually occurs under abiotic stress conditions, such as high or low temperature, poor nutrition or UV light. This phenomenon might be an aspect of the species preservation mechanism. Stress-induced flowering was described in studies on *Pharbitis nil* (Wada *et al.*, 2010a), *Perilla frutescens* var. *crispa* (Wada *et al.*, 2010b) and *Lemna paucicostata* (Shimakawa *et al.*, 2012). The influence of SA on flower development was first observed in 1965 (Lee and Skoog), when it was reported that the application of between 4 and 64  $\mu\text{M}$  SA (optimum of 32  $\mu\text{M}$ ) promoted flower bud formation in *Nicotiana* callus. Exogenous SA has been determined to be a flower-inducing factor in *Lemna gibba* G3 under noninductive photoperiodic conditions (Cleland, 1974, 1978; Cleland and Ajami, 1974; Kandeler, 1985). However, the concentration of endogenous benzoic acid (SA analogue) was determined in several *Lemna* species, including plants in both vegetative and flowering stages, by Fujioka *et al.* (1983). The

results did not reveal a difference in the benzoic acid concentration between the vegetative and generative stages of the plants. Therefore, it is possible that endogenous benzoic acid, and possibly endogenous SA, does not regulate the photoperiodic-induced flowering of this species. As such, it is possible that SA is necessary but not sufficient to induce flowering.

Nevertheless, a possible explanation for the flowering delay observed in our study might be the influence of the possibly elevated SA concentration in the transgenic lines on ethylene synthesis. Ethylene is involved in multiple aspects of floral development, from flower initiation to senescence. It has been shown that ethylene advances the transition from vegetative growth to flowering, among other species, in *Arabidopsis thaliana* (Ogawara *et al.*, 2003). A similar effect was observed in the *Bromeliaceae* family, *Plumbago indica*, mango and lychee (Abeles *et al.*, 1992). In 1988, Bleecker *et al.* showed that ethylene-insensitive mutants of *Arabidopsis* exhibited delayed flowering. Therefore, ethylene is a plant hormone that is considered to play a role in the transition from vegetative to reproductive growth or in floral development after flower bud differentiation. It has been shown that SA has an influence on ethylene biosynthesis in several studies by Leslie and Romani (1986, 1988), Romani *et al.* (1989) and Huang *et al.* (1993). SA has an inhibitory effect on the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene by suppressing the activity of ACC oxidase. Although it was observed that a low concentration of SA in carrot suspension cultures promoted endogenous ethylene biosynthesis (Nissen, 1994), Srivastava and Dwivedi (2000) reported that a high concentration of SA ( $>10^{-4}$  M) inhibited the synthesis of endogenous ethylene in banana fruits. It was demonstrated that SA interfered with ethylene synthesis or its accumulation by blocking the ACC oxidase (in pear suspension cultures; Szalai *et al.*, 2000) or by inhibiting ACC synthase transcript accumulation (in wounded tomato tissue; Li *et al.*, 1992). The inhibitory effect of SA on ethylene biosynthesis has been shown in several studies (e.g.



**Figure 4** Phenotypic traits of *Kalanchoë* control plants and 35S::AtMKS1 transgenic lines. (a) Plant height (cm); (b) internode length (cm); (c) number of nodes per plant; (d) number of days until first open flower (anthesis); (e) number of open flowers at the 50th day of measurements; (f) maximal number of flowers per plant; (g) inflorescence height (cm) after 6 weeks under short-day conditions; and (h) anthocyanin absorbance at 520 nm ( $\mu\text{mol/mL}$ ) in petals of *Kalanchoë* control and transgenic lines. Bars marked with different letters (a, b, c) are significantly different at  $P < 0.05$  by Tukey's multiple range test. Means  $\pm$  SD ( $n = 20$ ) are shown.

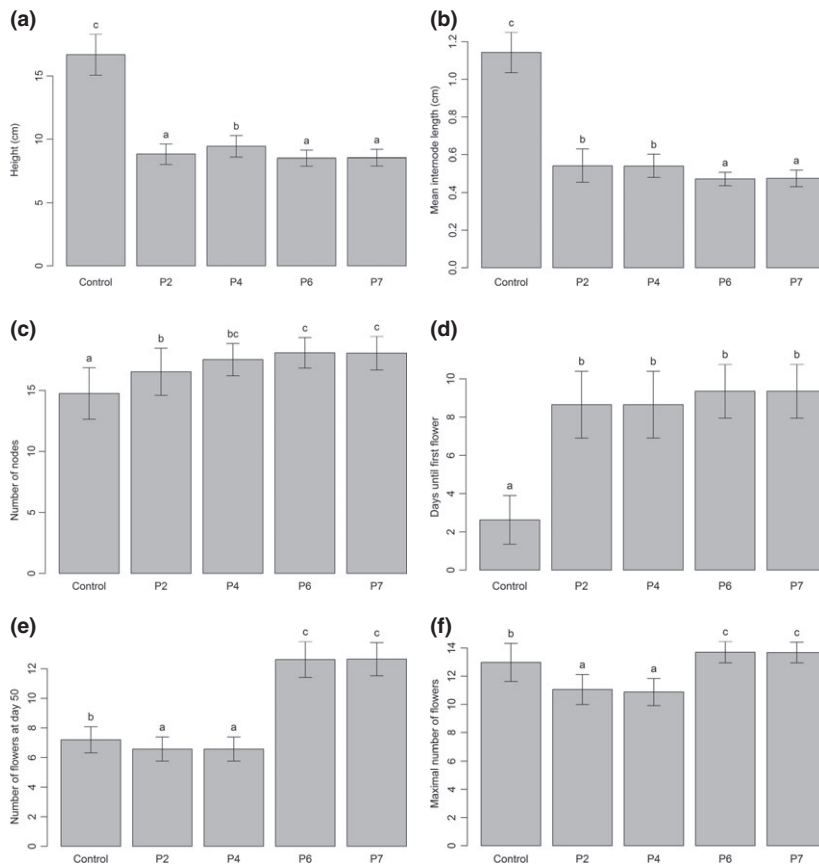
apple fruit discs (Fan *et al.*, 1996), carrot cell suspension cultures (Roustan *et al.*, 1990), mung bean hypocotyls, apple and pear fruit discs (Romani *et al.*, 1989) and pear cell cultures (Leslie and Romani, 1986, 1988). Another example was presented by Huang *et al.* (1993), who showed that SA inhibits the conversion of ACC to ethylene in detached rice leaves.

Therefore, under conditions in which the SA concentration is elevated, it might be assumed that the endogenous ethylene concentration is low, which diminishes the influence of ethylene on flower induction or on the vegetative to generative state transition. In the case of the transgenic lines investigated here, it is possible that a high SA concentration inhibits ethylene biosynthesis, which results in delayed flower induction.

#### Anthocyanin concentration in *Kalanchoë* petals

The petals of all *Kalanchoë* transgenic lines had significantly higher concentrations of anthocyanin than the petals of the nontransgenic control plants. Clone K1 had the highest concentration (average absorbance of extracts at 520 nm = 1.4)

(Figure 4h). Clone K2 had an average  $A_{520}$  value of 0.8, and clones K3 and K4 had an average  $A_{520}$  value of 0.5. These results appear to be correlated with the transgene copy number in the different lines, because clones K1, K2 and K3 have 4, 2 and 1 copy of the transgene, respectively. The anthocyanin concentration in clone K1 plants was approximately four times higher than that in control plants and was also clearly visible to the naked eye (Figure 3b). It has been shown that the application of SA to *Vitis vinifera* cell suspension cultures can enhance anthocyanin synthesis (Saw *et al.*, 2010). Similar results were reported by Sudha and Ravishankar (2003) in *Daucus carota*, where SA treatments were found to enhance *in vitro* anthocyanin biosynthesis in callus cultures. The increase in anthocyanin production is suggested to be due most likely to the increase in cytoplasmic  $\text{Ca}^{2+}$ . Another study on callus cultures of *Rosa hybrida* cv. Pusa Ajay confirmed the positive effect of SA on anthocyanin synthesis (Ram *et al.*, 2013). Application of  $10^{-5}$  M SA to *Zingiber officinale* cv. Halia Bara resulted in an anthocyanin concentration of 0.442 mg/g dry weight, while anthocyanin was undetectable in nontreated control plants (Ghasemzadeh *et al.*, 2012). It is possible that



**Figure 5** Phenotypic traits of *P. hybrida* control plants and *35S::AtMKS1* transgenic lines. (a) Plant height (cm); (b) internode length (cm); (c) number of nodes per plant; (d) number of days until first open flower (anthesis); (e) number of open flowers at the 50th day of measurements; and (f) maximal number of flowers per plant. Bars marked with different letters (a, b, c) are significantly different at  $P < 0.05$  by Tukey's multiple range test. Means  $\pm$  SD ( $n = 20$ ) are shown.

high SA levels in *Kalanchoë* plants expressing high levels of *MKS1* transcripts might influence the anthocyanin concentration in the flower petals. Nevertheless, the flowers of transgenic *Petunia* plants did not differ morphologically from the flowers of control plants.

#### Phenotype evaluation and quantitative RT-PCR assay of *Petunia* with down-regulated *PhMKS1* expression

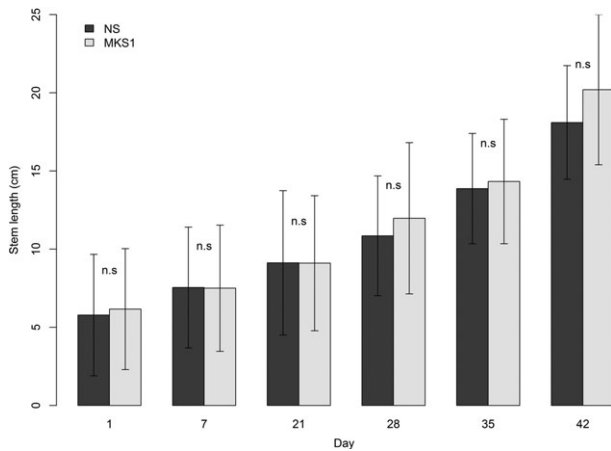
A comparison of the lengths of the main stems of plants infiltrated with *PhMKS1*-VIGS and NS-VIGS (NS –non-sense sequence) showed that the plants with decreased *MKS1* expression were slightly, but not significantly, taller than the NS-VIGS-treated plants (Figure 6).

However, qRT-PCR revealed significant differences in *PhMKS1* expression levels between *Petunia* plants treated with *PhMKS1*-VIGS and NS-VIGS constructs (Figure 7). The relative *PhMKS1* expression level was significantly reduced (between 4- and 8-times lower) in *PhMKS1*-VIGS-infiltrated plants when compared to NS-VIGS-treated plants in three independent qRT-PCR experiments. This result suggests that VIGS effectively reduced the expression of *PhMKS1* in *PhMKS1*-VIGS-treated *Petunia* plants; however, lower *PhMKS1* expression did not significantly influence the phenotype of the plants. In accordance with these observations, Andreasson *et al.* (2005) showed that the growth phenotypes of *Arabidopsis* mutants that express low levels of *MKS1* do not differ compared with the growth phenotypes of wild-type plants.

#### *Petunia* resistance to *Pseudomonas syringae* pv. *tomato*

Infected transgenic clones P2 and P4 were as sensitive as control plants to *Pseudomonas syringae* pv. *tomato* (Figure 8). On average, after 6 days, the plants exhibited sporadic pale spots,

and after 12 days postinoculation, all plants exhibited yellowish aureoles on the leaves. On day 16, most plants had yellow leaves with green edges. After 3 weeks, all plants had curled, yellowish leaves, especially on the lower part of the plant. On day 22 postinoculation, the plants began to show necrotic spots. *Petunia* lines P6 and P7 were more resistant to infection. The first class symptom—pale spots—was detectable approximately 2 weeks after inoculation in some of the plants. On day 19 postinoculation, yellowish aureoles could be observed in some plants. Some of the inoculated plants from lines P6 and P7 did not exhibit symptoms that were more severe than the first class (Figure 8). Plants from line P2 and P4 have one copy of the transgene integrated into the genome, and plants from lines P6 and P7 have four copies. Thus, our results indicate that plants with only one copy of *AtMKS1* and control plants react similarly to the infection. Accordingly, lines P6 and P7 most likely exhibit greater resistance to *Pst* infection as a result of increased *AtMKS1* expression due to the higher *AtMKS1* copy number in the genome. Higher *AtMKS1* expression in *Petunia* lines P6 and P7 may have led to the higher SA concentrations in local and systemic tissues and increased expression of the *PR1* genes; thus *Pst* infection in these lines did not lead to full disease development. According to Andreasson *et al.* (2005), the overexpression of *MKS1* in *Arabidopsis* results in increased resistance to biotrophic pathogens, which depend on live tissues and avoid triggering necrosis. *Arabidopsis* plants with constitutively up-regulated *MKS1* exhibit increased resistance to *Pst* infection, which agrees with our findings. SA-regulated *PR1* proteins may be directed primarily against apoplast-colonizing pathogens including biotrophic bacteria or certain fungi pathogens that form nutrient-absorbing structures (haustoria) and grow between the host cells while invading only small number of



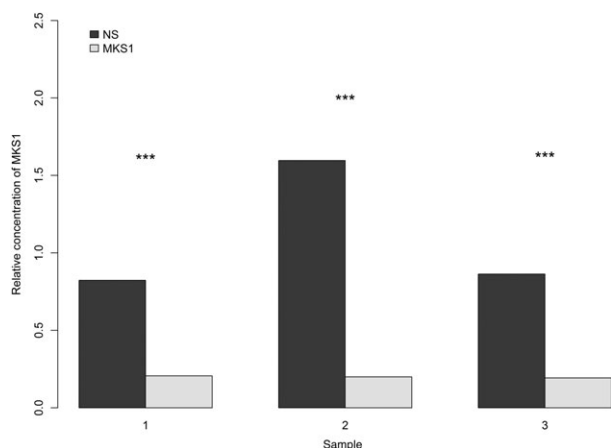
**Figure 6** Comparison of the stem length (cm) of *Petunia* plants infiltrated with *PhMKS1*-VIGS and NS-VIGS (non-sense sequence, not the influencing phenotype) vectors. The length of the stems was measured weekly. The mean  $\pm$  SD ( $n = 20$ ) is shown.

host cells (Oliver and Ipcho, 2004; Rico and Preston, 2008). Impaired SA synthesis or signalling in *Arabidopsis* mutants indicates that SA-dependent defences contribute to basal resistance against biotrophic pathogens (Thomma *et al.*, 2001).

## Experimental procedures

### Plant material

*Kalanchoë blossfeldiana* '1998-469' plants were provided by Knud Jepsen A/S (Hinnerup, Denmark) and *Petunia hybrida* 'Famous Lilac Dark Vein' plants were provided by Selecta Klemm GmbH & Co. KG (Stuttgart, Germany). Both species were



**Figure 7** Comparison of relative *PhMKS1* gene expression levels in *Petunia* plants treated with *PhMKS1*-VIGS and NS-VIGS (qRT-PCR was repeated three times for three of the same *PhMKS1*-VIGS-treated plants and three times for three the same NS-VIGS-treated plants). Significance code: \*\*\* $P < 0.001$  by log-transformation and a two-factorial analysis of variance. The fold change in the expression of *PhMKS1* was calculated relative to the untreated sample as a control after normalization to the *CYP* gene. The expression level in untreated samples is defined as 1 ( $n = 3$  in all experiments).

introduced and maintained in *in vitro* culture conditions as described by Gargul *et al.* (2013).

### Gene constructs and plant transformation

A binary vector containing the *AtMKS1* sequence was kindly provided by Professor John Mundy (Dept. of Biology, University of Copenhagen, Denmark). The construct was based on the pCAMBIA1301 sequence (<http://www.cambia.org/daisy/cambia/2046/version/1/part/4/data/pCAMBIA1301.pdf?branch=main&language=default>), in which the fragment with the *GUS* sequence was replaced by the *AtMKS1* sequence (Andreasson *et al.*, 2005). The vector was introduced into the *Agrobacterium tumefaciens* strain GV3101. Bacterial preparation, explant inoculation, co-cultivation and selection of transgenic plants were performed as described by Gargul *et al.* (2013). Transgenic lines of both species were acclimatized in a greenhouse under the following conditions: 16-h light and 8-h dark at 22 °C/18 °C for *Kalanchoë*, and 8.5-h light and 15.5-h dark at 22 °C/18 °C for *Petunia*.

### DNA isolation, PCR and Southern blot

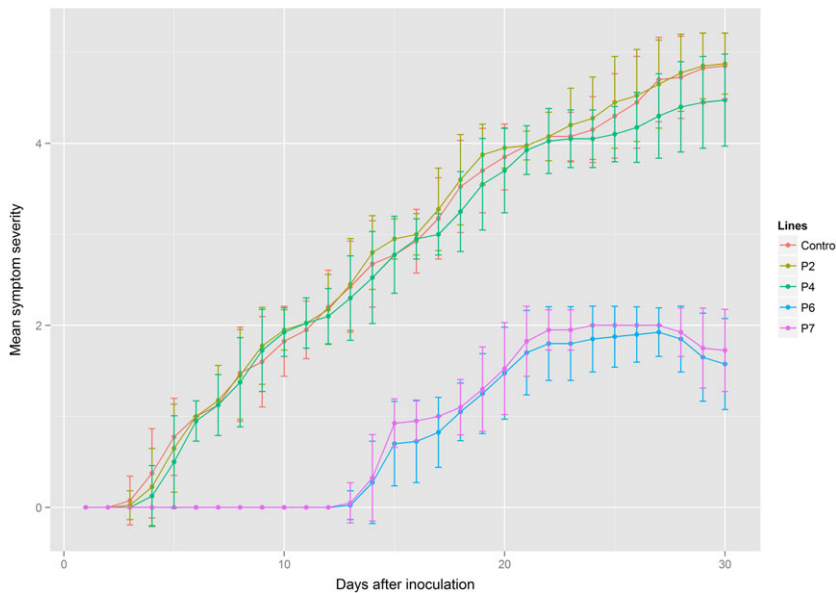
Genomic DNA from the transgenic lines and control plants of both species was isolated using the SeqLab Kit (Sequence Laboratories, Göttingen, Germany) according to the manufacturer's protocol. The PCR for screening the transgenic lines was performed as described by Gargul *et al.* (2013) using the *AtMKS1*-570 primer pair (Table 1). DNA from the following lines was digested with *Bam*HI (*Kalanchoë*) and *Hind*III (*Petunia*) as described by Gargul *et al.* (2013): *Kalanchoë* control; *Kalanchoë* transgenic lines K1, K2, K3 and K4; *Petunia* control; and *Petunia* transgenic lines P2, P4, P6 and P7. Southern blots were performed as described by Sriskandarajah *et al.* (2007). A digoxigenin-labelled probe targeting the *AtMKS1* gene was constructed using the *AtMKS1*-570 primer pair (Table 1) to amplify the *AtMKS1* gene from the pCAMBIA vector according to the manufacturer's protocol (Roche Applied Science Co., Mannheim, Germany). Hybridization, posthybridization and visualization of the hybridized fragments were performed as described by Sriskandarajah *et al.* (2007).

### Phenotype evaluation

Transgenic lines of *Kalanchoë* and *Petunia* were multiplied as cuttings with the same number of nodes. The height of the stems and the number of nodes were measured after the cuttings established a well-developed root system. *Kalanchoë* plants were maintained under long-day conditions, and *Petunia* plants were maintained under short-day conditions (described above) for stem measurements. The measurements were performed weekly for 5 weeks. To induce flowering, *Kalanchoë* plants were transferred to short-day conditions and *Petunia* plants were transferred to long-day conditions in the greenhouse. The observations began after anthesis of the first flower and were made daily for 50 days. The inflorescence stem length was measured once on the 35th day of observation. The growth and flowering habit of the transgenic lines were measured on two independent occasions with 20 plants per line.

### Anthocyanin concentration measurements in *Kalanchoë* petals

Petal material from 2-week-old flowers of control and transgenic lines was obtained for the anthocyanin extraction. Five milligrams of petal tissue was mixed with 1 mL of extraction solution [1%



**Figure 8** Severity of *Pseudomonas syringae* pv. *tomato* symptoms over time in *Petunia* control plants and transgenic lines P2, P4, P6 and P7. Observations were made daily for 30 days starting from infiltration. Symptom severity was ranked based on five stages, as described in the text.

HCl (37%) diluted in methanol]. The extraction was performed by shaking at 120 rpm for 30 min at 22 °C. The absorption was measured at 520 nm (Nielsen *et al.*, 2005) using a SmartSpec 3000 Spectrophotometer (BioRad, Hercules, CA).

#### Virus-induced gene silencing

A TRV-based vector system (Liu *et al.*, 2002; Ratcliff *et al.*, 2001) was used to investigate the effect of *MKS1* gene silencing on the growth habit of *Petunia hybrida* 'Fantasy Blue'. A *Petunia MKS1* fragment was amplified from *Petunia* cDNA using the *PhVIGS*-264 primer pair (Table 1). The primers were constructed based on a *Petunia* cDNA sequence obtained from a database (Sol Genomic Network; <http://solgenomics.net>) that was homologous to *Arabidopsis MKS1* (AT1G21326.1). The amplified *Petunia hybrida MKS1* (*PhMKS1*) fragment was cloned into a p-GEM-T Easy vector (Promega Co., Madison, WI). Subcloned cDNA fragments were removed from the p-GEM-T Easy vector by digestion with the *EcoRI* enzyme (Thermo Scientific/Fermentas, Vilnius, Lithuania) and ligated into the pTRV2 vector. The pTRV2 vectors were transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation and selected on LB media containing rifampicin (25 µg/mL) and kanamycin (50 µg/mL). The assisting vector pTRV1 was transformed into *A. tumefaciens* strain GV2260 and selected in the same medium. Harvested *A. tumefaciens* cultures were resuspended in 10 mM MgCl<sub>2</sub> with 150 µM acetosyringone and equal volumes of pTRV2- and pTRV1-containing cultures, which were then mixed. *Petunia* plants with a well-established root system previously grown under *in vitro* conditions were acclimatized to short-day conditions in the climate chamber. These plants were then used for *Agro*-infiltration by injecting the mixed bacterial cultures into the abaxial side of the leaf. All of the fully developed leaves on each plant were infiltrated. The experiments were performed twice with 20 plants per treatment. Plant height was measured weekly for 5 weeks after infiltration. After 5 weeks, the tips of selected *Petunia* plants were removed for RNA isolation. To serve as a noneffect non-sense sequence (NS), another TRV-RNA2 vector was used that contained a 280 bp fragment of the β-glucuronidase (*GUS*) sequence. For the control experiment, a phytoene desaturase (*PDS*) gene isolated from *N.*

*tabacum* was used as a reporter that caused leaf photo-bleaching. The TRV-based pTRV1 and pTRV2 vector constructs were kindly provided by Dr. Merete Albrechtsen, faculty of Agricultural Sciences, University of Aarhus.

#### RNA isolation, RT-PCR and quantitative RT-PCR assay

Total RNA was isolated from all *Kalanchoë* and *Petunia* transgenic lines and also from *Petunia* plants subjected to VIGS treatment as described by Gargul *et al.* (2013). For transgenic plants, RT-PCR was performed using the *AtMKS1*-570 primer pair (Table 1) to detect transgene expression. *KbPP2* (*Kb* protein phosphatase 2; acc. number: KC782950) (for *Kalanchoë*) and *CYP* (cyclophilin; Mallona *et al.*, 2010) (for *Petunia*) were used as housekeeping genes to evaluate the cDNA quality. RNA was isolated from randomly chosen *Petunia* plants infiltrated with *PhMKS1*-VIGS and NS-VIGS bacterial suspensions. The tissue used for extraction was selected from the youngest part of the shoot, including the three youngest leaves of the shoot. First-strand cDNA synthesis was performed as described by Gargul *et al.* (2013). To quantify mRNA levels between *Petunia* treated with the *PhMKS1*-VIGS vector and *Petunia* treated with the NS-VIGS vector, qRT-PCR assays were performed. Quantitative RT-PCR was performed using the Rotor Gene 3000 real-time thermal cycler (Corbett Life Science Co./Qiagen, Sydney, Australia). The reaction mixture had a final volume of 20 µL and contained the following: 0.5 ng of cDNA template, 0.15 mM each dNTP (Jena Bioscience, Jena, Germany), 0.25 µM each *PhVIGS*-134 or *CYP* primer (Table 1), 2 U of DCSHot DNA Polymerase (DNA Cloning Service, Hamburg), 10 mM TRIS HCl, 50 mM KCl, 2 mM MgCl<sub>2</sub> and SYBR Green (Roche Applied Science Co.). Eight minutes of incubation at 95 °C were followed by 45 cycles of 10 s at 94 °C, 30 s at 60–70 °C, and 30 s at 72 °C. To normalize the samples, the *CYP* expression levels (Table 1) were detected concomitantly with *PhMKS1*-VIGS- or NS-VIGS-treated samples. The PCR amplification specificity was checked by performing a melting curve analysis (from 70 to 94 °C) following the final PCR cycle. The PCR conditions were optimized for high amplification efficiency, and the data analysis was performed using Rotor Gene software (6.1.81). The relative quantification of the transcript abundance of target genes in individual plant



samples was determined using the  $2^{-\Delta\Delta CT}$  method. A total of three independent repetitions of the qRT-PCR reaction were performed for three independent, randomly chosen plant samples. Each sample was represented by three reaction tubes (biological replications) during the complete qRT-PCR run. Major changes in gene expression relative to that in control plants were calculated for each sample replicate (Livak and Schmittgen, 2001).

### *Pseudomonas syringae* pv. *tomato* tolerance test

A virulent strain of *P. syringae* pv. *tomato* was provided by the laboratory of Professor Kerstin Wydra (Tropenzentrum, Georg-August-Universität, Göttingen). Tests were conducted on *Petunia* control and transgenic lines P2, P4, P6 and P7. Bacterial suspensions were prepared as described by Hartmann (2008). The suspensions were adjusted to  $OD_{660} = 0.06$ , which corresponded to approximately  $10^7$  cells/mL. Well-rooted *Petunia* cuttings were inoculated by diluting the suspension 10 times and spraying it on the abaxial side of the six youngest well-developed leaves with a compressed-air-operated glass-sprayer (Ochs, Göttingen-Lenglern, Germany) until water-soaked spots appeared. Symptom observations were made daily for 30 days after infiltration and were classified as follows: 0, no symptoms; 1, sporadic pale spots; 2, yellowish aureoles; 3, yellowish leaf with a green edge; 4, completely yellow curled leaf; 5, necrotic spots.

### Statistical methods

The statistical analysis of the transgenic line phenotype evaluation was performed as previously described by Gargul *et al.* (2013). Relative expression values were log-transformed and a two-factorial analysis of variance was used. The *Pseudomonas syringae* infection symptom severity of 30 days indices were compared between clones using an exact (permutation-based) version of the Wilcoxon rank-sum test, and the resulting *P*-values were adjusted for multiple comparisons using the Holm method. The statistical analysis was performed using R 2.12.1 (R Development Core Team, 2010).

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