Stem growth regulation by molecular breeding in *Kalanchoë blossfeldiana* and *Petunia hybrida*

Von der Naturwissenschaftlichen Fakultät der

Gottfried Wilhelm Leibniz Universität Hannover

zur Erlangung des Grades

Doktorin der Gartenbauwissenschaften

(Dr. rer. hort.)

genehmigte Dissertation

von

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geboren am 15. Februar 1982 in Kraków, Polen

2017

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Tag der Promotion: 22.03.2016

Abstract

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Production of attractive, compact and healthy ornamental plants requires the usage of chemical growth regulators and pesticides. Clarification of molecular mechanisms underlying the plant growth and defense responses, which took place over the last few decades could lead to novel and more environmentally friendly strategies in the production of ornamentals. Growth regulators mostly target the metabolism of the gibberellic acid, which is a phytohormone responsible mostly for longitudinal plant growth. Its biosynthetic pathway and the involved enzymes are well studied in model plants. Another plant hormone, salicylic acid, induces systemic acquired resistance which helps the plant to tolerate pathogen attacks.

To minimize the usage of growth retardants and pesticides, biotechnological strategies towards the production of compact and pathogen tolerant ornamentals were applied in the economically important species *Kalanchoë blossfeldiana* and *Petunia hybrida*. To this end, a key enzyme of gibberellic acid biosynthesis, gibberellin 2-oxidase (GA_2ox) from *Nicotiana tabacum*, and MAP4-kinase substrate 1 (MKS1) from *Arabidopsis thaliana*, were ectopically expressed, respectively. The constitutive over-expression of *NtGA$_2$ox* resulted in a significant reduction of stem growth and increased chlorophyll content in leaves of both species. In contrary virus induced gene silencing of *GA$_2$ox* in *Petunia* resulted in significant increase of the stem growth. Phenotypic analysis of *AtMKS1*-transgenic *Kalanchoë* and *Petunia* plants revealed a reduction of stem length, but in contrast to *NtGA$_2$ox* plants, also a significant increase of anthocyanin content in the petals. Silencing of *MKS1* did not influence the phenotype of *Petunia*. Additionally, *AtMKS1*-transgenic *Petunia* plants showed elevated tolerance towards *Pseudomonas syringae* pv. tomato.

Flower shape and size of *NtGA$_2$ox* and *AtMKS1 Kalanchoë* and *Petunia* resembled the appearance of wild type flowers. However, the transgenic plants exhibited delayed flowering, which is an undesired quality characteristic of ornamental plants. To prevent this negative side effect but preserve the effect on stem growth, a stem-specific expression of both genes was desired. To this end, the *NtGA$_2$ox* was expressed in *Kalanchoë* and *Petunia* under *PAL1* promoter with deleted BOX-I isolated from *Pisum sativum*. The over-expression of *GA$_2$ox* driven by the modified promoter resulted in significant reduction of the stem growth but also deformation of the leaf blades in both species.

Keywords: salicylic acid, gibberellic acid, compact phenotype

Regulation des Sprosswachstums durch molekulare Züchtung in *Kalanchoë blossfeldiana* und *Petunia hybrida*

Die Produktion attraktiver und gesunder Zierpflanzen ist abhängig vom Einsatz chemischer Wachstumsregulatoren und Pestiziden. Die Aufklärung molekularer Mechanismen in Pflanzen ermöglicht die Entwicklung neuer, umweltfreundlicher Strategien zur Produktion von Zierpflanzen. Primäres Ziel der verwendeten Wachstumsregulatoren ist das Pflanzenhormon Gibberellinsäure, welches für das Längenwachstum pflanzlicher Organe verantwortlich ist. Der Biosyntheseweg der Gibberellinsäure und die involvierten Enzyme konnten in Modellpflanzen vollständig aufgeklärt werden. Das Pflanzenhormon Salicylsäure hingegen ist maßgeblich an der Induktion der Systemisch Akquirierten Resistenz (SAR) beteiligt und hilft der Pflanze Angriffe pathogener Organismen zu tolerieren.

Um den Gebrauch chemischer Wachstumsinhibitoren und Pestizide zu minimieren, wurden biotechnologische Strategien zur Produktion kompakter, pathogen-toleranter Zierpflanzen am Beispiel der wirtschaftlich relevanten Arten *Kalanchoë blossfeldiana* und *Petunia hybrida* entwickelt. Dafür wurde ein Schlüsselenzym der Gibberellinsäure-Biosynthese, die Gibberellin 2-Oxidase (GA_2ox) aus *Nicotiana tabacum* und zum anderen das MAP4-Kinase Substrat 1 (MKS1) aus *Arabidopsis thaliana* exprimiert. Die konstitutive Überexpression der *NtGA$_2$ox* resultierte in einer signifikanten Reduktion des Sprosswachstums und einem erhöhten Chlorophyllgehalt in den Blättern beider Arten. Umgekehrt konnte ein verstärktes Längenwachstum von *Petunia* Sprossen durch Herabregulation der *GA$_2$ox* mittels Virus-induzierten *Gene Silencing* beobachtet werden. Die phänotypische Analyse der *AtMKS1* exprimierenden *Kalanchoë* und *Petunia* erwies eine Reduktion des Sprosswachstums, aber im Gegensatz zu den *NtGA$_2$ox* Pflanzen, eine signifikante Erhöhung des Anthocyan-Gehalts in den Blütenblättern. Zudem wiesen die *AtMKS1-Petunia* eine erhöhte Toleranz gegenüber *Pseudomonas syringae pv. tomato* auf. Virus-induziertes *Gene Silencing* von *MKS1* hatte keinen Einfluss auf den Phänotyp von *Petunia*.

Form und Größe der *NtGA$_2$ox* und *AtMKS1 Kalanchoë* und *Petunia* Blüten glich optisch dem Wildtyp, die Blüte setzte aber verspätet ein. Um diesen negativen Effekt zu kontrollieren, wurde eine Spross-spezifische Expression der Gene angestrebt. Dafür wurde die *NtGA$_2$ox* unter Kontrolle eines modifizierten Phenylalanin Ammonium Lyase (PAL1) Promoters aus *Pisum sativum* exprimiert, was zu einer signifikanten Reduktion des Sprosswachstums aber auch zu einer Deformation der Blattspreiten von *Kalanchoë* und *Petunia* führte.

Schlüsselwörter: Salicylsäure, Gibberellinsäure, Kompakten Phänotyp

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List of abbreviations

35S	35S constitutive promoter from Cauliflower Mosaic Virus
BA2H	benzoic-acid-2-hydroxylase
BAK1	BRI-associated kinase 1
BRI1	brassinosteroid-insensitive 1
CPS	ent-copalyl diphosphate synthase
ent-CDP	ent-copalyl diphosphate
dBI	deletion BOX-I stem-specific promoter
dex	dexamethasone
flg22	flagellin
FLS2	flagellin-sensitive 2
GA	gibberellin
GA2ox, 2ox	gibberellin 2-oxidase
GA3ox, 3ox	gibberellin 3-oxidase
GA13ox, 13ox	gibberellin 13-oxidase
GA20ox, 20ox	gibberellin 20-oxidase
GAI	GA insensitive
GID1	gibberellin insensitive dwarf 1
GGDP	geranylgeranyl diphosphate
ICS	isochorismate synthase
IPL	isochorismate pyruvate lyase
Kb	*Kalanchoe blossfeldiana*
KO	ent-kaurene oxidase
KAO	ent-kaurenoic acid oxidase
KS	ent-kaurene synthase
MAPK	mitogen-activated protein kinase
MEKK1	mitogen-activated protein kinase kinase kinase 1
MKK1	mitogen-activated protein kinase kinase 1
MKK2	mitogen-activated protein kinase kinase 2

MKS1	map kinase 4 substrate 1
MPK4	mitogen-activated protein kinase 4
NPR1	non-expresser of PR genes 1
PAL	phenylalanine ammonia lyase
PAMP	pathogen-associated molecular pattern
Ph	*Petunia hybrida*
SA	salicylic acid
SAR	systemic acquired resistance
WRKY33	transcription factor
XET	xyloglucan endotransglycosylase
XTH	xyloglucan endotransglucosylase/hydrolase

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Introduction

Introduction

Kalanchoë blossfeldiana (*Kb*) and *Petunia hybrida* (*Ph*) are two popular ornamental plant species representing different growth habit. *Kalanchoë* is a potted succulent indoor plant and *Petunia* is a bedding herbaceous outdoor plant. *Kalanchoë* is one of the most economically important indoor plant in Europe. According to Flora Holland the sale was assessed at 84 million plants with a turnover of EUR 60 million in 2014 (Flora Holland, 2015). Sale of *Petunia* was estimated at 11 million plants with a turnover of EUR 8 million in 2014 (Flora Holland, 2015).

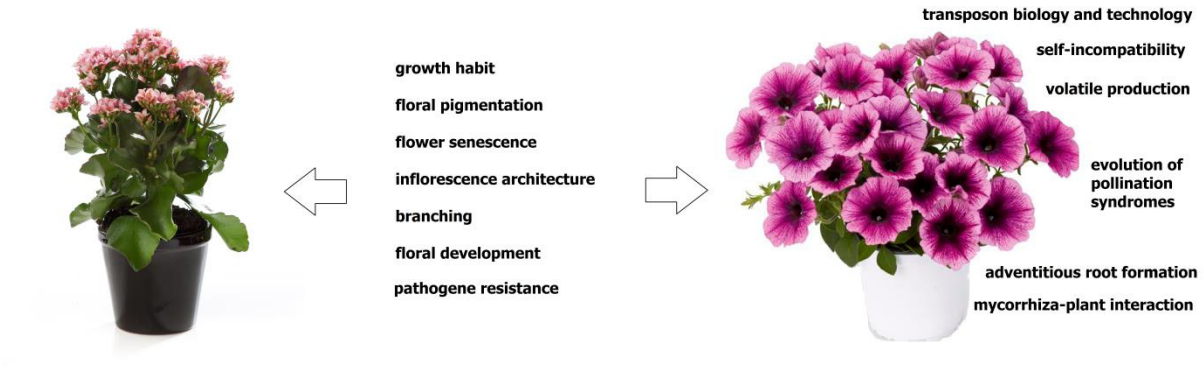

Fig. 1. Focused subjects in *Kalanchoë* and *Petunia* molecular breeding.

Compact growth habit next to the flower structure, scent and petal color modification, flowering time prolongation, postharvest quality, abiotic stress tolerance improvement and disease resistance is one of the most desired features in ornamental potted plants breeding. Dwarf-like growth habit is not only valued by the customers regarding its appeal but also growers value compact plants which take less space in production and are easier to handle in transport. Traditional methods for inducing compact growth habit relay on the manipulation of factors like light, watering and temperature and often also pinching for elimination of apical dominance. Along with traditional factors, growers also apply chemical growth retardants which mostly interfere with the gibberellin (GA) biosynthesis. These chemicals are inhibitors of *ent*-kaurene oxidase. Their mode of action is based on inhibiting the oxidation of *ent*-kaurene into *ent*-kaurenoic acid in the biosynthesis of GA (Rademacher, 2000). Most popular growth retardants in ornamental plants production are i.e.: paclobutrazol (Bonzi, Cambistat, Cutdown, Downsize, Florazol, Paclo, Paczol, Piccolo, Profile, Shortstop, Trimmit), daminozide (B-Nine, Dazide, Compress) or chlormequat chlorid (Cycocel, Citadel,

E-Pro). Those chemicals control stem length, but do not improve branching and also cause delayed flowering. The possible negative impact to the environment or human health of chemical growth retardants have been discussed in the previous years in Europe (Fujimoto et al., 1997; Andersen et al., 2002). At present ancymidol (A-Rest, Abide), uniconazole (Sumagic, Concise) and flurprimidol (Topflor, Mastiff, Cutless, Legacy) previously used chemical growth retardants are no longer approved by the European Commission for usage in EU (http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=activesubstance.selection&language=EN). The molecular breeding approach in growth retardation has been studied over last few years as an alternative strategy to the application of chemicals. Mostly the molecular manipulation was targeted at gibberellin balance. Also positive results were obtained with introduction of *rol* genes or alcohol treatments (Christiansen et al., 2008; Topp et al., 2008; Mibus et al., 2014).

The objective of this study is to establish a molecular breeding method for compact growth in both *Kalanchoë* and *Petunia*. In order to reach the objective both species were submitted to *Agrobacterium*-mediated transformation with binary vectors containing *gibberellin 2-oxidase* isolated from *Nicotiana* driven by 35S constitutive and *dBI* stem-specific promoters and also *map kinase substrate 1* gene isolated from *Arabidopsis* driven also by 35S constitutive promoter.

This thesis consists of the overview of the used growth retardation methods and description of the phytohormonal influence on the growth and development of the plants. The experimental work and results are presented in three published papers, one submitted manuscript and in addition three conference papers and one poster. And last part of the thesis considers discussion of presented results and possible further outlook.

Literature review

Overview

Plant hormones are classically defined as small, mobile compounds that, in trace quantities, influence growth and development in tissues distant from the sites of synthesis (Davies, 2004). Plant hormones regulate mainly the speed of growth, differentiation and development but also control the process of reproduction and stimulate defensive responses. All aspects of growth and development are regulated by genes and are under hormonal control, either via changes in hormone concentrations in response to changes of gene transcription, or via the hormones themselves as regulators of gene transcription (Davies, 2010). Presented here research was focused on the investigation of phenotypic changes in *Kalanchoë* and *Petunia* transgenic plants with altered balance of gibberellins or salicylic acid.

Gibberellin

Gibberellins are a family of over 136 compounds based on the *ent*-gibberellane structure. The name gibberellin was derived from fungus *Gibberella fujikuroi*, now reclassified as a *Fusarium fujikuroi*. It was first described in Japan in the late 19t century. The disease caused by the fungus in rice generates symptoms of excessive seedling elongation and sterility (Hori, 1898). In the 1950s the cooperation of the research between USA, UK and Japan lead to the isolation and structural determination of the active compound from the fungus and was named gibberellin A_3 (GA_3). It was discovered that GA_3 have significant effects on plant growth, which induce bolting and flowering in rosette species and enables to rescue dwarf mutants of pea and maize. After observing similar effects with application of plant extracts it has been speculated that gibberellins are endogenous plant metabolites. This hypothesis was confirmed in 1958 by isolation of gibberellin A_1 (GA_1) from immature seeds of runner bean (MacMillan and Suter, 1958). The breakthroughs in the understanding of gibberellin function on molecular level had place in the 1990s and 2000s when mutant *GAI* cDNA in *Arabidopsis* and its mutant allele *gai* (*GA-insensitive*) was cloned (Peng et al., 1997). It has been shown that gibberellins act to relieve growth repression by GAI which is a member of the DELLA subgroup. DELLA proteins act in partnership with transcription factors to regulate gene expression. The understanding of early events in GA perception and action was possible after isolation of the GID1 (gibberellin insensitive dwarf 1) GA receptor and when it has been observed that GA induces DELLA protein degradation via the ubiquitination-proteasome pathway (Wang and Deng, 2014).

The biosynthesis is initiated in the chloroplasts and subsequently involves membrane and cytoplasmic stages. They are synthesized in young tissues of the shoots and seeds from glyceraldehyde-3-phosphate, via isopentenyl diphosphate. GAs are synthesized from geranylgeranyl diphosphate (GGDP). It is a C-20 precursor for diterpenoids. In plants there are three classes of enzymes which are required for the synthesis of bioactive GAs from GGDP: terpene synthases, cytochrome P450 monooxygenases and 2-oxoglutarate–dependent dioxygenases. It has been shown that methylerythritol phosphate pathway in the plastid supply most of the isoprene units to GAs in *Arabidopsis* seedlings, while there is a minor addition from the cytosolic mevalonate pathway (Kasahara et al., 2002). The *ent*-copalyl diphosphate synthase and *ent*-kaurene synthase are located in plastids and involved in the conversion of GGDP to the tetracyclic hydrocarbon intermediate *ent*-kaurene (Aach et al., 1997, Helliwell et al., 2001, Sun et al., 1994, 1997). Subsequently *ent*-kaurene is converted to GA_{12} by two cytochrome P450 monooxygenases. *ent*-Kaurene oxidase, which is located in the outer membrane of the plastid catalyzes the sequential oxidation on C-19 to produce *ent*-kaurenoic acid, which is converted to GA_{12} by another *ent*-kaurenoic acid oxidase located in the endoplasmic reticulum (Nelson et al., 2004, Helliwell et al., 2001). GA_{12} is converted to the GA_4 bioactive form through oxidations on C-20 and C-3 by GA 20-oxidase (GA20ox) and GA 3-oxidase (GA3ox) which are 2-oxoglutarate–dependent dioxygenases (Fig. 2). Those enzymes are assumed to be localized in cytosol. GA20ox produces C_{19}-GAs using GA_{20}-GAs as substrates by catalyzing the sequential oxidation of C-20. The inactive precursors GA_9 and GA_{20} are converted to active GA forms by introduction of the 3β-hydroxyl group. The precursor for the bioactive GA_1 is GA_{53} is produced in the 13-hydroxylated pathway where GA_{12} is substrate for GA13ox (Fig. 2).

Gibberellins are responsible for stem growth by stimulating cell elongation and division, bolting in response to long day conditions, induction of seed germination and together with light signals induction of flowering or fruit setting and development. Gibberellins induce transcription of genes engaged in these processes. For instance, expression of genes encoding expansins or xyloglucan endotransglycosylases (XET) in elongating internodes of *Arabidopsis* or rice is regulated by gibberellins (Cho and Kende, 1997, Uozu et al., 2000, Xu et al., 1996). Expansins are proteins which induce cell wall loosening by disrupting the polysaccharide adhesion. XET is involved in xyloglucan reorganization by cleaving and re-ligating xyloglucan polymers in the cell wall what influences the call wall plasticity. The gibberellin perception and signal transduction pathway transform the GA signal into shifts of gene expression and changes of plants morphology.

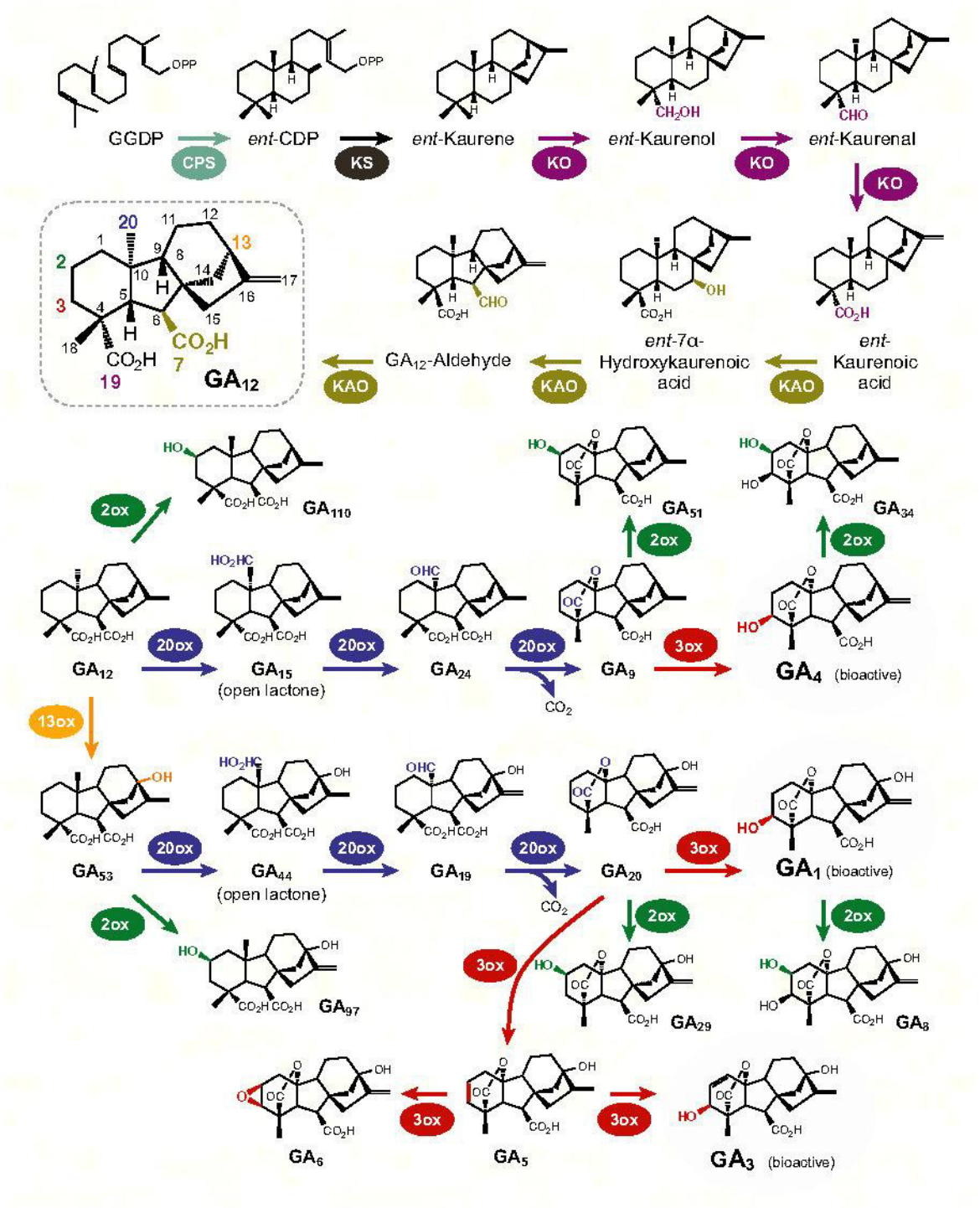

Fig. 2 Gibberellin biosynthesis and deactivation pathways in plants. 2ox - GA 2-oxidase; 3ox - GA 3-oxidase; 13ox - GA 13-oxidase; 20ox - GA 20-oxidase; GGDP - geranylgeranyl diphosphate; ent-CDP - ent-copalyl diphosphate; CPS - ent-copalyl diphosphate synthase; KS - ent-kaurene synthase; KO - ent-kaurene oxidase; KAO - ent-kaurenoic acid oxidase (by Yamaguchi, 2008).

GAs have tetracyclic ent-gibberellane or 20-nor-ent-gibberellane skeletons with 4 rings.
The biological activity for growth stimulation of GAs depends on their structure. GA must not possess a 2β-hydroxyl group but must be a C-19-GA with a 4,10 lactone and a carboxylic acid group on C-6 to be bioactive. It also has a 3β-hydroxyl group or some other functionalization at C3 position (GA_1 and GA_4). 2β-hydroxylation is a deactivating mechanism, therefore a GA which has some functionality at C-2 that prevents 2β-hydroxylation might have enhanced activity. Since GA_3 and GA_7 possess a 1, 2 double bond, they are not substrates for the 2β-hydroxylating enzyme. The structural requirements for florigenic activity might differ from those for stem elongation (Yamaguchi, 2008) . It has been shown in *Lolium* that GA_5 and GA_6 are more active in enhancing flowering than GA_1 or GA_4. On the other hand, GA_1 and GA_4 promote stem growth in this genus (King et al., 2003)
The deactivation of bioactive hormones in plants is important for keeping the homeostasis and effective regulation. Gibberellins are mostly deactivated by 2β-hydroxylation, a reaction which is catalyzed by GA 2-oxidases. Overexpression of GA deactivating genes has been recognized as an alternative to the growth retardants (Phillips, 2004). Different examples of the manipulation in GA homeostasis have been described and discussed in presented papers and discussion part of this thesis.

Map Kinase Cascade and Map Kinase 4 Substrate 1

Different environmental and endogenous factors continuously influence plant growth and development. For protection against negative influence plants were evolutionary forced to develop effective mechanisms at the molecular, cellular and organ levels to be able to defend their basic metabolism, cell structures, membranes and transport processes. Plants upon environmental stimuli have developed systems of fast and specific reactions to protect, defense or alter their development which are based on a cellular responses based on a cascade of events. The cascade starts with the molecular perception and recognition of the stimuli. It generates the signal which is then transmitted through signaling pathways what results in the activation of specific effectors responsible for the stimulation of the particular molecular reaction (Soropy and Munshi, 1998; Møller and Chua, 1999). The effective signaling system based on the signal transduction is determined by protein phosphorylation and dephosphorylation that is regulated by protein kinases and phosphatases. It is a system which interconnects the sensing system with gene expression processes according to the physiological cell status. One of such protein kinase signaling pathways is mitogen-activated

protein kinase cascade (MAPK). It is activated upon signal perception, and the signal transmission control cellular processes such as cell division, differentiation and responses to the environmental stress factors. (Bögre et al., 2000; Wrzaczek and Hirt, 2001; Tena et al., 2001; Jonak et al., 2002; Šamaj et al., 2004a, b; Nakagami et al., 2005; Colcombet and Hirt, 2008). MAPKs act in units, which are signal transducers of extracellular signals in eukaryotic cells. The basic unit of a MAPK pathway consists of three functionally interlinked serine/threonine protein kinases, which are categorized according to the amino acid which are phosphorylated upon activation. For full activation of MAPKs the phosphorylation of both tyrosine and threonine residues is necessary. The phosphorylation is catalyzed by a dual-specificity of MAPKK. Their activation occurs by phosphorylation of their serine and threonine residues by MAPKKKs (Robinson and Cobb, 1997). The signal is accepted at the plasma membrane, then the transmittance, intensification and targeting of the signal takes place in the cytoplasm. Effective cell responses depend on the specificity of the substrate which activate the MAPK and synergistic and/or antagonistic MAPK pathways activated by external stimuli. MAPK signal transduction pathways enable cells to have efficient control of gene expression, metabolism, cytoskeletal structure and dynamics by the influence on activation of transcription factors, cytoplasmatic enzymes or cytoskeletal proteins (Garrington and Johnson,1999; Bögre et al., 2000; Jonak et al., 2002; Nakagami et al., 2005). The specificity and mechanism in response to different stimuli and in different cell types are based on the correct localization and accessibility of the MAPKs in the cell. A particular class of anchoring and scaffold proteins brings together specific kinases for precise activation and control the subcellular localization of signaling complexes within the cell (Garrington and Johnson, 1999). The scaffolding enables the specificity of MAPK signaling because of creating multi-enzyme complexes formed by several signal molecules in certain compartments which facilitate rapid passage of the signal through the cascade and restrain unwanted crosstalk (Whitmarsh and Davis, 1998). The termination of the activated MAPKs is catalyzed by different protein phosphatases: serine/threonine-specific phosphoprotein phosphatases and metal-ion-dependent protein phosphatases, phosphotyrosine phosphatases and dual-specificity phosphatases (Farkas et al., 2007). The dephosphorylation resets the pathway to the initial state.

Plant innate immunity consists of two interconnected sections. One is a pathogene-associated molecular pattern (PAMP)-triggered immunity, which is based on the recognition of molecular signatures of pathogens and activates downstream MAP kinase cascade and transcription of defense genes. Another is an effector-triggered immunity, which is driven by

plant pathogen resistance proteins which recognize specific pathogen-derived effectors (Chisholm et al., 2006). Both of these systems activate local and systemic defense responses which are called systemic acquired response (SAR) which is regulated by plant hormones such as salicylic acid (SA) (Fu and Dong, 2013). Such responses to the pathogen attacks involve large-scale transcriptional reprograming, which depend also on those of transcription factor families like WRKY (Eulgem, 2005; Ryu et al., 2006; Naoumkina et al., 2008).

MAP kinase 4 substrate 1 (MKS1) is a VQ protein which directly interacts with MAP kinase 4 (MPK4). The VQ proteins are a group of transcription regulators which interact with transcription factors in order to modulate downstream gene expression. VQ proteins can also form regulator modules such as WRKY-VQ-MPK what enables fine-tune gene transcription. These proteins can either positively or negatively regulate different responses in plant immunity, abiotic stress or plant growth and development. The expression of the affiliated genes is altered in response to internal or external signals (Jing and Lin, 2015). Activation of MPK4 triggers the phosphorylation of VQ21 i.e. MKS1 at multiple serine residues. The MKS1 phosphorylation causes WRKY33 and MKS1 to deasociate from MPK4 (Andreasson et al., 2005; Caspersen et al., 2007) (Fig. 3). It has been reported that upon challenge with the *Pseudomonas syringae* or upon elicitation by the microbe-associated molecular pattern flg22, the WRKY33 transcription factor is released from the WRKY33-MKS1-MPK4 trimeric complex and subsequently binds to the *PAD3* promoter (Andreasson et al., 2005; Qiu et al., 2008). *PAD3* gene encodes for cytochrome P450 monoxygenases which are required for the synthesis of the antimicrobial phytoalexin camalexin (Petersen et al., 2008).

Andreasson et al. (2005) showed that constitutive overexpression of *MKS1* gene in *Arabidopsis* leads to increased tolerance to *Pseudomonas*, increased levels of *PR1* transcripts and almost 4-fold increase in SA levels. Accumulation of the defense hormone SA and secretion of the antimicrobial PR (pathogenesis-related) proteins indicate of the SAR activation in the plant.

Salicylic Acid

Salicylic acid influences plant growth and development, photosynthetic machinery, flowering, enzyme activities and membrane permeability. SA is a plant hormone which can either inhibit germination or increase seed vigor. The contradictory effects depend on the employed SA concentrations (Rajou et al., 2006, Shakirova, 2007). Soaking grains of wheat

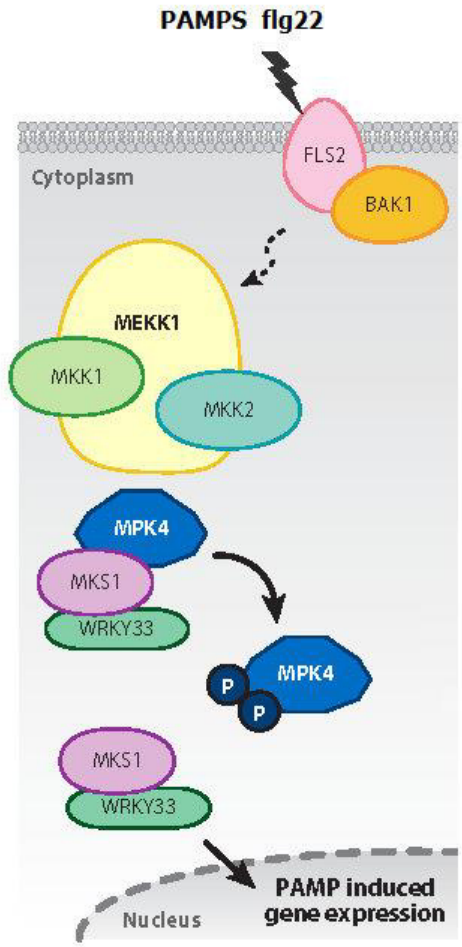

Fig. 3 MAPK cascades in PAMP-triggered immunity. PAMP – pathogen-associated molecular pattern, flg22 – flagellin, FLS2 – flagellin-sensitive 2, BAK1 – BRI-associated kinase 1, MEKK1 – mitogen-activated protein kinase kinase kinase 1, MKK1 – mitogen-activated protein kinase kinase 1, MKK2 – mitogen-activated protein kinase kinase 2 , MPK4 – map kinase 4, MKS1 – map kinase 4 substrate 1, WRKY33 – transcription factor (by Suarez-Rodriguez et al., 2010).

in 10^{-5} M of SA resulted in increased number of leaves and fresh and dry mass per plant (Hayat et al., 2005). Also growth promoting responses were observed in barley or maize seedlings sprayed with SA (Pancheva et al., 1996, Khodary, 2004). Miura et al. (2010) has shown that xyloglucan endotransglucosylase/hydrolase (*XTH*) genes transcription levels that encode enzymes which are involved in cell wall loosening and cell expansion (Rose et al., 2002), are strongly reduced in *cpr5* (*constitutive expressor of PR gene 5*) *Arabidopsis* mutants (Bowling et al., 1997). This *Arabidopsis* mutant has constitutively high levels of SA and exhibit dwarfed phenotype. Similar correlations between increased SA levels and compact growth habit was also observed in *acd6-1* (*accelerated cell death 6-1*; Rate et al.,

1999), and *agd2* (*aberrant growth and death 2*; Rate and Greenberg, 2001) *Arabidopsis* mutants. Examples of the growth-inhibiting SA influence in *Arabidopsis* are presented in Table 1.

Salicylic acid is a plant hormone which is biosynthesized in two ways from the amino acid phenylalanine. SA biosynthesis requires chorismate as a primary metabolite and might be based on two distinct enzymatic routes (Wildermuth, 2006; Chen et al., 2009) (Fig. 4). The synthesis process might be localized either in cytoplasm or alternatively in chloroplast. The phenylalanine route which takes place in cytoplasm is based on the conversion of chorismate-derived phenylalanine to trans-cinnamic benzoic acid (BA), and hydroxylation of its aromatic ring, which is catalyzed by benzoic-acid-2-hydroxylase (BA2H) leads to the formation of SA (Leon et al., 1993; Lee et al., 1995). It has been reported that the presence of hydrogen peroxidase is required for the conversion of BA to SA, what stimulates the activity of BA2H (Chong et al., 2001) (Fig. 4). This process was observed in heat-treated pea plants, salt-stressed rice plants and ozone-exposed tobacco leaves (Ogawa et al., 2005; Sawada et al., 2006; Pan et al., 2006). The chloroplast-localized SA synthesis is a two-step reaction catalyzed by isochorismate synthase and isochorismate pyruvate lyase from chorismate via isochorismate (Wildermuth et al. 2001) (Fig. 4). This process is observed in plants upon pathogen infections and UV- or ozone-exposed *Arabidopsis*, *Nicotiana benthamiana* and tomato (Ogawa et al., 2005; An and Mou, 2011). SA plays role in plant defense-related actions against infections caused by various pathogens. SA is a molecule which plays role in defense-related long- and short-distance signaling in plants. SA is involved in the SAR. It is a mechanism of induced defense which confers long lasting protection against viruses, bacteria, fungi and oomycetes (Fu and Dong, 2013). In the 1961 it has been shown by Ross that the pathogenic attack on older leaves causes the development of resistance in younger leaves. In his study he shows that the infection of a local lesion tobacco host with Tobacco Mosaic Virus induced local and systemic resistance to a secondary TMV inoculation. It has been shown that isochorismate pathway is a major source of SA during SAR. SA influences the positive regulator protein NPR1 (non-expresser of *PR* genes 1), which moves to the nucleus where the protein binds with transcription factors of genes involved in defense response.

Examples of *Arabidospsis* genotypes with increased SA levels exhibiting reduced growth phenotype

Transgene/ mutation	Gene name	Gene function	References
acd5	*accelerated cell death 5*	Ceramide kinase	Greenberg et al., 2000 Liang et al., 2003
acd6	*accelerated cell death 6*	Encodes for a protein with putative ankyrin and transmemebrane regions	Rate et al., 1999
acd11	*accelerated cell death 11*	Sphingosine transmembrane transporter	Brodersen et al., 2002 and 2005
agd2	*abberant growth and death 2*	Member of ARF GAP domain	Rate and Greenberg, 2001 Vanacker et al., 2001 Song et al., 2004
atsr1	*Arabidopsis thaliana signal responsive 1*	Ca2+/calmodulin-binding transcription factor (CAMTA3)	Du et al., 2009
cpr1	*constitutive expresser of PR 1*	Unknown	Bowling et al., 1994 Scott et al., 2004 Mateo et al., 2006
cpr5	*constitutive expresser of PR 5*	Unknown	Bowling et al., 1997 Mateo et al., 2006
cpr6	*constitutive expresser of PR 6*	Unknown	Clarke et al., 1998
dnd1	*defense, no death 1*	Cyclic nucleotide-gated ion channel (AtCNGC2)	Yu et al., 1998 Clough et al., 2000 Mateo et al., 2006
dnd 2	*defense, no death 2*	Cyclic nucleotide-gated ion channel (AtCNGC4)	Yu et al., 2000 Jurkowski et al., 2004
lsd6	*lesions simulating disease 6*	Unknown	Weymann et al., 1995 Mateo et al., 2004
mks1	*MAP kinase 4 substrate 1*	Substrate of MAP kinase 4	Andreasson et al., 2005

mpk4	*MAP kinase 4*	Regulates salicylic acid- and jasmonic acid/ethylene-dependent responses via EDS1 and PAD4	Brodersen et al., 2006
ssi1	*supressor of SA insensivity 1*	Unknown	Shah et al., 1999

Tab.1 Examples of *Arabidospsis* genotypes with increased SA levels exhibiting reduced growth phenotype.

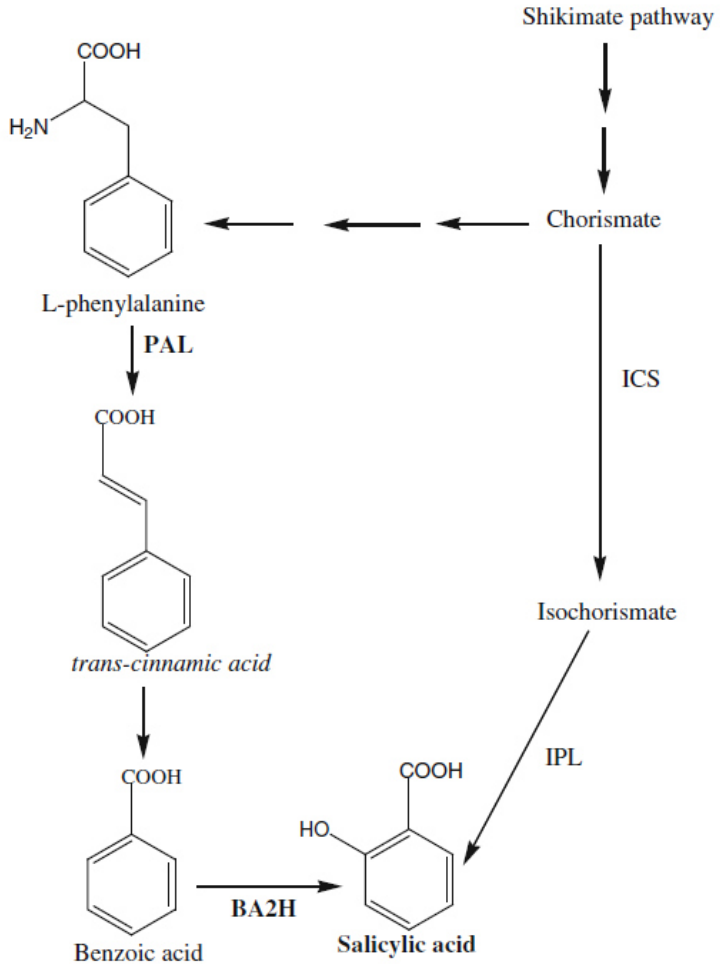

Fig. 4 SA biosynthesis pathways. Abbreviations: PAL - phenylalanine ammonia lyase, ICS - isochorismate synthase, IPL - isochorismate pyruvate lyase, BA2H - benzoic-acid-2-hydroxylase (by Bandurska, 2013).

Taxonomy, morphology and habitat of *Kalanchoë blossfeldiana* and *Petunia hybrida*

Kalanchoë blossfeldiana (von Poellnitz) belongs to the *Crassulaceae* family which is native to Madagascar. It is reported to be first introduced in Europe by Robert Blossfeld in 1932 in Potsdam (Broertjes and Leffring, 1972). It is a perennial, succulent plant which forms dense tufts to approx. 40 cm. *Kalanchoë* has erect, little-branched stems with fleshy, petiolate, dark green, shining leaves which are arranged along the stem in pairs and the flowers consist of small star-shaped florets produced in a short-day conditions. The peduncle might elongate up to 15 cm. (Eggli, 2003)

Petunia hybrida (Hook.) Vilm. belongs to the *Solanaceae* family. It is a hybrid derived from *P. integrifolia* and *P. axillaris* which are endemic to South America with the most diverse species located in Brazil. It was first obtained by a British nurseryman Atkins of Northampton in 1834 (Sink, 1984). *Petunia hybrida* is mostly an annual plant with herbaceous stems. Leaves are elliptical with petiole with flat surface and margins. The inflorescences are sympodial with monochasial growth (Fries, 1911; Danert, 1958).

Methods of growth retardation for *Kalanchoë blossfeldiana*

It has been described in Larson et al. (1980) that most popular growth retardant used on *Kalanchoë* was B-Nine, which is applied 3-5 weeks after the start of the short day (SD) (8.5 h of light) exposition. And repeating treatment is applied 4-5 weeks after the first application. To induce branching and the development of flower buds the pinch of the growing tips of the stems up to the second or third leaf is practiced. After the branching induction, when the axillary shoots reach approx. 4 cm in length B-Nine is also applied. Ancymidol might be used as a foliar spray or more effective drench. The drench should be applied up to 4 weeks after potting (Schnabel and Carlson, 1976; Pertuit, 1973). According to Kai Lønne Nielsen, research and production manager at Knud Jepsen A/S (Denmark) different *Kalanchoë* cultivars require different set of treatments with chemical growth retardants. During the production process *Kalanchoë* are treated at least twice. First treatment applied early in the production to obtain a desired body shape, plants are treated with Cycocel Extra [active

ingredient: Chlormequat (2-chloroethyl) trimethylammonium chloride]. Second treatment with Alar or B-9 (active ingredient: Daminozide), in combination with Cycocel Extra required before flowering is applied for reduction of the flower stem elongation.
Also after development of the flower heads but before elongation of the peduncles it is possible to periodically withhold water in between irrigations, what results in shorter stems of the plants (Anonymous, 1976). During flowering *Kalanchoë* plants should be grown under SD conditions but high intensity of light, what also contributes to the compact phenotype (Laurie et al., 1968). The practice of removing the terminal inflorescence before the elongation of the flowering stem and after 4 to 5 weeks from SD exposition leads also to the more compact plants and development of additional inflorescence. This treatment results in plants shorter up to 10 cm in comparison to untreated plants, and flowering is delayed several days (Love, 1976 a and b; Rathmell, 1970). For tall cultivars pinching of vegetative terminal shoot performed 1-2 weeks after the start of SD exposition results in a well-formed floral display without delayed flowering. Pinching of the vegetative terminal shoot abolishes the apical dominance what induces the development of auxiliary shoots and increases the number of potential inflorescences (Anonymus, 1976).

Methods of growth retardation and increased branching for *Petunia hybrida*

Petunia is a rapid growing plant. Nevertheless producers prefer plants which are not stretched, narrow and well branched what minimizes stem entanglement and facilitates dense packing for transportation. *Petunia* requires good light conditions starting from the spring to assure fast flowering and prevent stretching. *Petunia* produces flowers faster in a long day (LD) photoperiod (16 h of light), but it can flower in any conditions. Photoperiods with 13 or more hours of light result in early flowering and taller, unbranched plants. Short day conditions of approx. 8 to 10 hours of light results in delayed flowering, retarded elongation of the main stem and induction of lateral branching. Together with low light availability the temperature should be also lowered down. The response to the photoperiod is correlated with the temperature. In the average daily temperatures lower than 20 °C *Petunia* plants are compact and well branched regardless of the photoperiod nevertheless they flower faster when exposed to the LD conditions. If the average daily temperatures are higher than 20 °C with short days *Petunia* plants have more branches than under LD conditions, but the

flowering is delayed. The most rapid flowering of *Petunias* is observed in LD conditions when the temperature is higher than 20 °C, however in these conditions stems are elongated, the lateral branching is restricted and the leaves are smaller. The correlation between temperature and photoperiod is most visible during spring production. In the beginning of spring *Petunia* is compact and the flower formation takes longer time. Further in the season together with the increase of the temperature and photoperiod stems of *Petunia* plants are more elongated and the flower formation time is shorter.

Francescangeli and Zagabria (2008) has presented results in *Petunia* Bravo F1 cultivar of paclobutrazol (2RS,3RS)-1-(4-chlorofenil)-4,4-dimetil-2-(1H-1,2,4-triazol-1-yl)pentan-3-ol) influence on height and flower formation. Depending on the investigated *Petunias* with different flower colour, the irrigation with concentration of 10 mg L^{-1} of paclobutrazol induced maximal growth retardation of 38 % in red-flowered plants to 56% in blue-flowered plants. There were some deleterious effects on number of flowers observed.

Producers often use anti-gibberellin based PGRs. Such chemicals control stem length but they don't improve branching and unfortunately delay flowering. In *Petunia* production at the point of the production when the temperature rises and the photoperiod length increases B-Nine is applied when the plants are approx. 5 cm in diameter (2500 to 5000 ppm). Subsequent application should be made 7 to 10 days later. Bonzi (15 to 50 ppm) is effective in a single application. Chemical growth retardants should not be applied after flower buds are visible (Kessler, 1998). Carey et al. (2007) has studied cytokinin based PGRs. Cytokinins increase cell division and improve growth and shoot formation (Leclerc et al., 2006; Nordström et al. 2004). Nonetheless, the increase in cytokinin concentration is usually observed after bud outgrowth has begun, therefore the timing of cytokinin action might be too slow to trigger bud outgrowth (Turnbull et al.,1997). Benzyladenine is a cytokinin which is a main ingredient of Exilis Plus, a chemical growth regulator which is used in apple production as an thinning fruit and increasing fruit size and agent. In *Petunia* the single foliar spraying of 80 ppm with Exilis Plus appeared to decrease the width of the plant but also increased the number of flowers per plant. Double application of 160 ppm Exilis Plus has decreased the width and increased the plant height in the free branching cultivars (Carey et al., 2007).

Compilation of publications

Plant Cell Tiss Organ Cult (2013) 115:407–418
DOI 10.1007/s11240-013-0372-5

ORIGINAL PAPER

Constitutive overexpression of *Nicotiana GA$_2$ox* leads to compact phenotypes and delayed flowering in *Kalanchoë blossfeldiana* and *Petunia hybrida*

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Received: 25 March 2013 / Accepted: 15 August 2013 / Published online: 27 August 2013

Abstract This work describes compact phenotypes of *Kalanchoë blossfeldiana* and *Petunia hybrida* plants harboring a constitutively overexpressed *gibberellin 2-oxidase* (*GA$_2$ox*) transgene. A *GA$_2$ox* gene from *Nicotiana tabacum* under the control of the Ca35S promoter was introduced into the pCAMBIA1303 plasmid. The cloning vector was introduced into leaf explants of *Kalanchoë* and *Petunia* via *Agrobacterium*-mediated transformation. Putative transformants were analysed for the presence, integration and expression of the transgene using polymerase chain reaction (PCR), reverse-transcription (RT)-PCR, and Southern blot analysis, respectively. Phenotypic evaluations revealed that the mean lengths of the *Kalanchoë* transgenic lines were two-fold shorter than those of wild-type control plants, although the mean numbers of nodes were similar. Moreover, the mean lengths of inflorescence stems of the *Kalanchoë* transgenic lines were almost three-fold shorter than those of the wild-type control plants. Similarly, the mean lengths of *Petunia* transgenic lines were four-fold shorter than those of the wild-type plants, except for a single line, while the mean numbers of nodes were either similar or higher in the transgenic lines than in the wild-type control plants. In transgenic lines of both *Kalanchoë* and *Petunia*, delayed flowering was observed with a mean of 24 days for *Kalanchoë* and a range of three to 12 days for *Petunia*. Although the flower morphology of the transgenic lines did not exhibit any differences from their respective wild-type control plants, transgenic lines of both species exhibited darker green pigmented leaves containing an approximately two-fold increase in chlorophyll contents over the wild-type control plants.

Keywords Compact growth · *GA$_2$ox* · *Gibberellin 2-oxidase* · Ornamental plants · Transgenic plants

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Introduction

Kalanchoë blossfeldiana (*Kb*) is an ornamental plant that belongs to the *Crassulaceae* family (Van Voorst and Arends 1982; Uhl 1948), and it has recently become a best-seller among flowering indoor potted plants. The number of *Kalanchoë* plants sold per year is approximately 77 million, generating revenues of EUR 55 million in 2012 (Flora Holland 2013). However, *Kalanchoë* plants produce long internodes and inflorescence stems during flower development, which highly decreases their ornamental value as a potted plant. To avoid this effect and fulfill the expectations of customers, chemical growth retardants are applied during production. Most chemical growth retardants inhibit the different enzymes involved in gibberellin (GA) biosynthesis. Although each cultivar of *Kb* is treated according to an individual program, all cultivars are treated twice during the production process. Cycocel Extra [active ingredient: Chlormequat (2-chloroethyl) trimethylammonium chloride] is mainly used early in the production process to shape the body of the plant, and Alar (B-9) (Daminozide), in combination with Cycocel Extra, is used late in the production process to reduce the elongation of the flower stems (research & production manager Kai

Lønne Nielsen; Knud Jepsen A/S—pers. comm.). The dwarfing effect of chemicals lasts only one flowering season (Rademacher 2000). Additionally, some growth retardants are highly hazardous to humans and have already been banned or restricted in the European Union (Andersen et al. 2002; Bhattacharya et al. 2010). For these reasons, molecular biological research aimed at reducing the length of plant stems has intensified. One research area is the direct genetic manipulation of GA metabolism. GA is a plant hormone that plays an essential role in plant growth, tissue differentiation and development (Hedden and Phillips 2000; Bhattacharya et al. 2010). It has been shown that GA-deficient mutants are usually much shorter than the wild-type plant. Furthermore, through its involvement in signal transduction pathways, GA, together with light signals, can induce flowering and the production of the inflorescence stem. GA metabolism is complex. GA 2-oxidases (GA_2ox), which belong to the group of 2-oxoglutarate-dependent dioxygenases, are responsible for the deactivation of GA_1, GA_4 and their precursors into inactive molecules by 2-β-hydroxylation (Hedden and Phillips 2000; Yamaguchi 2008). In accordance with this phenomenon, it has been reported that the overexpression of *GA_2ox* transgenes resulted in dwarf phenotypes in *Solanum melanocerasum* (Dijkstra et al. 2008), *Solanum nigrum* (Dijkstra et al. 2008), *Nicotiana tabacum* (Ubeda-Tomas et al. 2006), and *Nicotiana sylvestris* (Lee and Zeevaart 2005). Similar results were observed for *Oryza sativa* (Sakamoto et al. 2001; Sakai et al. 2003; Sakamoto et al. 2003), *Arabidopsis thaliana* (Thomas et al. 1999; Hedden and Phillips 2000; Wang and Li 2005) and a *Populus tremula* × *Populus alba* hybrid (Busov et al. 2003).

The aim of the present study was to investigate the effects of the molecular manipulation of GA metabolism in *K. blossfeldiana* and *Petunia hybrida*. *Petunia hybrida (Solanaceae)* was introduced as a model plant to compare the treatments in two different species. *Petunia* is also an important outdoor ornamental plant. The number of plants produced per year is approximately 14 million, generating a revenues of EUR 9 million in 2012 (Flora Holland 2013). *Kalanchoë* and *Petunia* differ in their growth habits. *Kalanchoë* is produced as an indoor succulent, and *Petunia* is an herbaceous outdoor bedding plant. The flowering inductions also differ. Flowering in *Kalanchoë* plants is induced when subjected to short day (SD) conditions, whereas *Petunia* flowering in the present work was induced using long day (LD) conditions. The up-regulation of the *GA_2ox* gene from *N. tabacum* in *Kalanchoë* and *Petunia* plants was investigated using reverse transcription PCR. The transgene copy number was determined for transgenic clones in both species, and the phenotypes of all modified plants were characterised under greenhouse conditions.

Materials and methods

Plant material

Plants of *K. blossfeldiana* cv. '1998-469' were provided by Knud Jepsen A/S (Hinnerup, Denmark). This cultivar has a very elongated growth habit. *Petunia hybrida* 'Famous Lilac Dark Vein' plants were provided by Selecta Klemm GmbH & Co. KG (Stuttgart, Germany). *Kalanchoë* leaf tissue was grown in culture, and the regenerated shoots were maintained in vitro as described by Ilczuk et al. (2009). Young *Petunia* leaves were harvested from greenhouse-grown plants, surface sterilised by soaking in 2 % (w/v) sodium hypochlorite with 0.1 % Tween 20 for 15 min, and subsequently rinsed three times in sterile water. The leaves were cut into 2 pieces through the midrib to obtain 0.5 × 0.5 cm rectangular explants. The *Petunia* explants were placed on a solid regeneration medium based on half strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 20 g L^{-1} sucrose and 8.0 g L^{-1} plant agar (Duchefa, Haarlem, The Netherlands), 1 mg L^{-1} 6-benzyladenine (BA), and 0.2 mg L^{-1} 1-naphthaleneacetic acid (NAA). The pH of this medium was adjusted to 5.7 before autoclaving. *In vitro* shoot regeneration of both species was performed on 6 cm diameter Petri dishes. After 3 to 4 weeks, the *Petunia* shoots were harvested and transferred to a root-inducing MS medium containing half strength MS macro- and microelements supplemented with 20 g L^{-1} sucrose and 8.0 g L^{-1} plant agar (Duchefa, Haarlem, The Netherlands). Shoot regeneration and root induction for both species were performed in a growth chamber at 24 °C with a 16 h photoperiod of 32 μmol m^{-2} s^{-1} illumination.

Vector construction and plant transformation

The *GA_2ox* gene sequence of *N. tabacum* was cloned (AB125232.1) in front of the 35S constitutive promoter within a pCAMBIA1303 vector (Cf. Web references) by replacing the GFP-GUS reporter gene fusion. The T-DNA contains a hygromycin gene for selection in plants under a duplicated 35S promoter. The vector also contains an *Npt II* gene under the 35S promoter for bacterial selection. The modified construct was cloned in the *Agrobacterium tumefaciens* strain GV3101.

A bacterial suspension was prepared from a 100 μL frozen glycerol culture in 25 mL of LB medium (10 g L^{-1} bacto-tryptone, 5 g L^{-1} bacto-yeast extract, 10 g L^{-1} NaCl, pH 7.5) supplemented with 50 mg L^{-1} kanamycin and 25 mg L^{-1} rifampicin and cultured for 24 h at 28 °C with shaking (250 rpm). The bacterial suspension (20 mL) with an $OD_{600} = 0.6$ was pelleted at 4,000 rpm for 15 min at 4 °C. The supernatant was discarded, and the pellet was

Table 1 Primer names, sequences and amplicon characteristics

Primer name	Target sequence	Primer pair	Sequence (5′–3′)	Amplicon size (bp)
NtGA$_2$ox-571	*NtGA$_2$ox* in T-DNA in genomic DNA	Forward	CCAAAGACAACTGCAAACCA	571
		Reverse	TGCTCACCAAATCCAATCAA	
NtGA$_2$ox-306	*NtGA$_2$ox* in cDNA made from mRNA	Forward	ATGGATCAGCACTTCTCCAAAG	306
		Reverse	GAGAATGTATTCGACCCAACCA	
NtGA$_2$ox-438	*NtGA$_2$ox* in T-DNA in pCAMBIA plasmid (probe synthesis)	Forward	CCCCTTGTCCTGAGATTCAA	438
		Reverse	TGAGGCTGCAATTTTCTCAA	
HptII	*HptII* in T-DNA in genomic DNA and in pCAMBIA plasmid (probe synthesis)	Forward	GATGTTGGCGACCTCGTATT	579
		Reverse	GATGTAGGAGGGCGTGGAT	
pCAMBIA	pCAMBIA non-T-DNA	Forward	GCTGAAGCCAGTTACCTTCG	800
		Reverse	GAAAGCTGCCTGTTCCAAAG	
KbPP2	*Kalanchoë protein phosphatase 2 gene*	Forward	GGGGAAGTTTGCTGCTACTG	255
		Reverse	GCAACCATGTAACGAACACG	
CYP	*Petunia cyclophilin gene*	Forward	AGGCTCATCATTCCACCGTGT	111
		Reverse	TCATCTGCGAACTTAGCACCG	

re-suspended in 20 mL of half strength liquid MS medium. The solution was supplemented with 150 μM acetosyringone. Young leaf explants from the in vitro grown plants of *Kalanchoë* and *Petunia* were cut into 5 × 5 mm squares and submerged in the bacterial solution for 10 min. The explants were blotted dry on sterile filter paper and placed on co-cultivation media (shoot-inducing media supplemented with 15 mg L^{-1} acetosyringone) for 2 days. After co-cultivation, the explants were transferred to shoot-inducing medium (described in the plant material section) supplemented with hygromycin and cefotaxime. The effective concentration level of hygromycin as a selection agent was evaluated for both species. The experiment was repeated three times for each species, with the use of 50 explants and 50 shoots per species per treatment and a range of hygromycin concentrations between 1 and 10 mg L^{-1} for both the shooting and rooting phases. For *Kalanchoë* shoot induction, the selection medium was supplemented with 5 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime. For *Kalanchoë* root induction, the selection medium was supplemented with 8 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime. The selection medium for *Petunia* shoot induction was supplemented with 3 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime, and the selection medium for root induction was supplemented with 5 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime. The explants were transferred into fresh media every 3 weeks. Regenerated shoots were cut from the explant and transferred to the selection media for root induction. The shoots were transferred into fresh media every 3 weeks until they were 4 to 8 cm high and produced a well-developed root system. Then, the plants were acclimatised and transferred to greenhouse conditions.

PCR, RT-PCR and Southern blot hybridisation

The regenerated plants were examined for the presence of T-DNA in the genomic DNA by PCR with the NtGA$_2$ox-571 and HptII primers (Table 1). Genomic DNA was isolated with a Seqlab Kit (Sequence Laboratories, Göttingen, Germany) according to the manufacturer's protocol. PCR amplification was conducted in a thermocycler (Biometra, Göttingen, Germany) under the following conditions: 2 min at 95 °C for the initial denaturation, followed by 40 cycles consisting of 30 s at 95 °C for denaturation, 1 min 20 s at 68 °C (NtGA$_2$ox and HptII primers) or 58 °C (pCAMBIA primers) for annealing, 2 min for extension and a final extension for 10 min at 72 °C. To exclude bacterial contamination, which would result in the false positive amplification of the vector DNA, a PCR with primers amplifying sequences from the backbone of the vector was performed (pCAMBIA primers, Table 1). For Southern blot hybridisation, approximately 12–15 μg of DNA from the control plants, *Kalanchoë* lines K5, K7, K9, K13, K15 and K16, and *Petunia* lines P2, P13, P14, P15

and P16 were digested using 30 units of *BamH*I (*Kalanchoë*) or *Hind*III (*Petunia*) (Thermo Scientific/Fermentas, Vilnius, Lithuania) for 24 h, with an additional 15 units used for the next 24 h. The resulting DNA fragments were separated on agarose-gels and transferred to a membrane as described in Sriskandarajah et al. (2007). A DIG-labelled (digoxigenin) probe was prepared by PCR according to the manufacturer's protocol (Roche Applied Science Co. Mannheim, Germany) using the plasmid with the inserted construct containing the $NtGA_2ox$-438 primer pair (*Kalanchoë*) and the HptII primer pair (*Petunia*) (Table 1). The hybridisation, post-hybridisation and an estimation of the visualised fragments were performed as described in Sriskandarajah et al. (2007).

For RT-PCR verification of *$NtGA_2ox$* expression, total RNA was isolated for all transgenic lines of *Kalanchoë* and *Petunia*. The RNA was isolated from 30 mg of ground plant material using the Invisorb Spin Plant RNA Mini Kit (Invitek & Co./STRATEC Molecular, Birkenfeld, Germany) according to the manufacturer's protocol. The RNA concentration and quality was measured using a NanoDrop 2000 (Thermo Fisher Scientific Inc. Waltham, MA; USA). To remove genomic DNA, the samples were treated with 1 µL of rDNAse (DNA-*free*™ DNase Treatment and Removal Reagents Kit, Ambion, CA, USA) in a thermocycler (Biometra, Gottingen, Germany) at 37 °C for 30 min and with another 1 µL of rDNAse for 20 min. The enzyme was deactivated by the Inactivation Reagent (DNA-*free*™ Kit, Ambion, CA, USA) according to the manufacturer's protocol. To evaluate the RNA quality after rDNAse treatment, 1 µL of total RNA was fractionated on a 1 % agarose gel, visualised by staining with ethidium bromide and compared with standard concentrations of kDNA (Thermo Scientific/Fermentas). To exclude possible genomic DNA residues in the RNA samples, PCR was performed using primer pairs for the housekeeping genes encoding protein phosphatase 2 (*KbPP2*, KC782950.1) and cyclophilin (*CYP*, Mallona et al. 2010), for *Kalanchoë* and *Petunia,* respectively (Table 1). Reverse transcription was performed using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific/Fermentas) with 3 µg of RNA and 20 pM random hexamer primer according to the supplied protocol. RT-PCRs were prepared using 0.5 ng of cDNA in a final volume of 20 µL containing 0.25 µM each $NtGA_2ox$-306 primer (Table 1), 0.15 mM each dNTP, 10 mM TRIS HCl, 50 mM KCl, 2 mM $MgCl_2$ and 1 U of DCS Polymerase (DNA Cloning Service, Hamburg, Germany). The reaction consisted of denaturation at 95 °C for 2 min, followed by 40 three-step cycles consisting of denaturation for 30 s at 95 °C, annealing at 68 °C for 1 min 20 s (for each primer pair), and extension at 72 °C for 2 min. A final 10-min extension step at 72 °C was also applied.

Phenotypic evaluation

Plants with a confirmed T-DNA integration event in the genome were multiplied using tip cuttings that contained the same number of internodes. After 2 weeks, when the cuttings developed a proper root system, the plants were subjected to weekly measurements. The total length and number of internodes were measured. *Kalanchoë* plants were kept under long day (16 h of light) conditions at 22 °C/18 °C (day/night), whereas *Petunia* plants were kept under short day conditions (8.5 h of light). Flowering was induced by the transfer of *Kalanchoë* to short day conditions and of *Petunia* to long day conditions. The plants were not chemically treated (conditioned) or trimmed either before or during the evaluation. Open flowers were counted daily. The inflorescence stems of *Kalanchoë* were measured once at 6 weeks after the anthesis of the first flower.

Chlorophyll determination

Chlorophyll measurements were made for both *Kalanchoë* and *Petunia*. Three 8-mm-diameter discs were excised from the center of the blade of the third leaf from the top of the plant using a cork borer. Each disc was weighed (fresh weight). The chlorophyll content was analysed according to Lichtenthaler (1987). Extraction was performed in 80 % (v/v) ethanol at 75 °C for 10 min. Absorption was measured using a SmartSpec™ 3000 Spectrophotometer (BioRad, Hercules, CA, USA) at 647, 664 and 700 nm. The chlorophyll content was calculated according to the equation:

$$\text{Chlorophyll a} + \text{b}(\mu\text{g/mL in extract}) = 5.24 \times (A_{664} - A_{700}) + 22 \times (A_{647} - A_{700})$$

where A is the absorbance at 647, 664 or 700 nm. The results were expressed as mg of chlorophyll per g of fresh leaf tissue weight.

Statistical analysis

The data of the phenotypic evaluation of vegetative growth and chlorophyll content were analysed by linear mixed models with replications, clones, times and clone-time interactions as fixed factors. To account for repeated measurements within the plants over time, the plant was included as a random effect, and an AR1 structure was used to model the correlation between the repeated measurements within the plant (Pinheiro and Bates 2000). The variables length and length/node ratio were log-transformed prior to analysis. After fitting the model, multiple comparison procedures (Hothorn et al. 2008) were used to compare the means of the clones to the means of the control plants for each variable at each time point, pooled over the two replications. In the bar charts, clones labelled by the same letter do not differ

significant at the 5 % level in the Tukey test. The data of the phenotypic evaluation of reproductive growth of transgenic lines were analysed using the Wilcoxon tests for the pairwise comparisons among clones, adjusting for multiple comparisons using the Holm-method. In the bar charts, clones labelled by the same letter do not differ significant at the 5 % level in the Holm adjusted Wilcoxon tests. The statistical analysis was performed with the program R 2.12.1 (R Development Core Team 2010) further relying on Hothorn et al. (2008) and the Piepho (2004).

Results and discussion

The aim of the present work was to perform a comparative study on the effects of increased *GA_2ox* expression in horticultural plants, with the goal of developing a method for creating a more compact plant that does not require chemical treatments. Both species were transformed with the same construct, in which the *GA_2ox* gene isolated from *N. tabacum* is controlled by the constitutive 35S promoter.

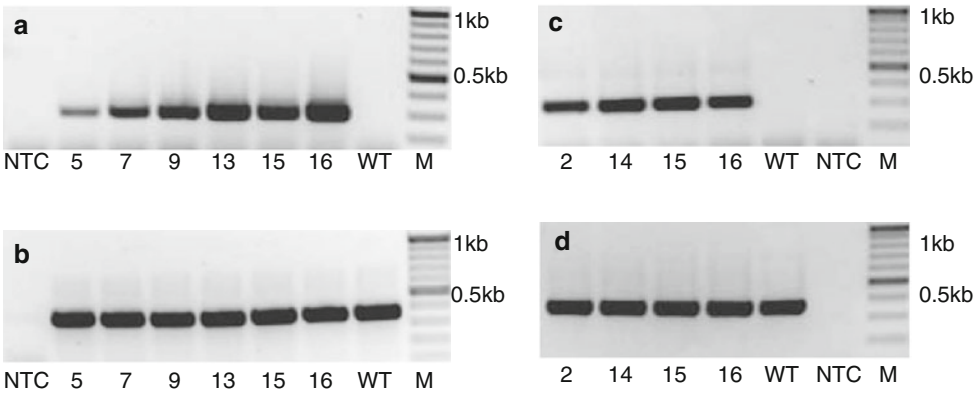


Fig. 1 RT-PCR of the transgenic *K. blossfeldiana* lines using the gibberellin 2-oxidase specific NtGA_2ox-306 primer pair (**a**) and the KbPP2 primer pair (**b**); RT-PCR of the transgenic *P. hybrida* lines using the NtGA_2ox-306 primer pair (**c**) and with the CYP primer pair (**d**) (Table 1). WT—non-transgenic control cDNA sample of the *Kalanchoë* (**a**, **b**) and *Petunia* (**c**, **d**) plants; NTC—non-template control; M—DNA ladder

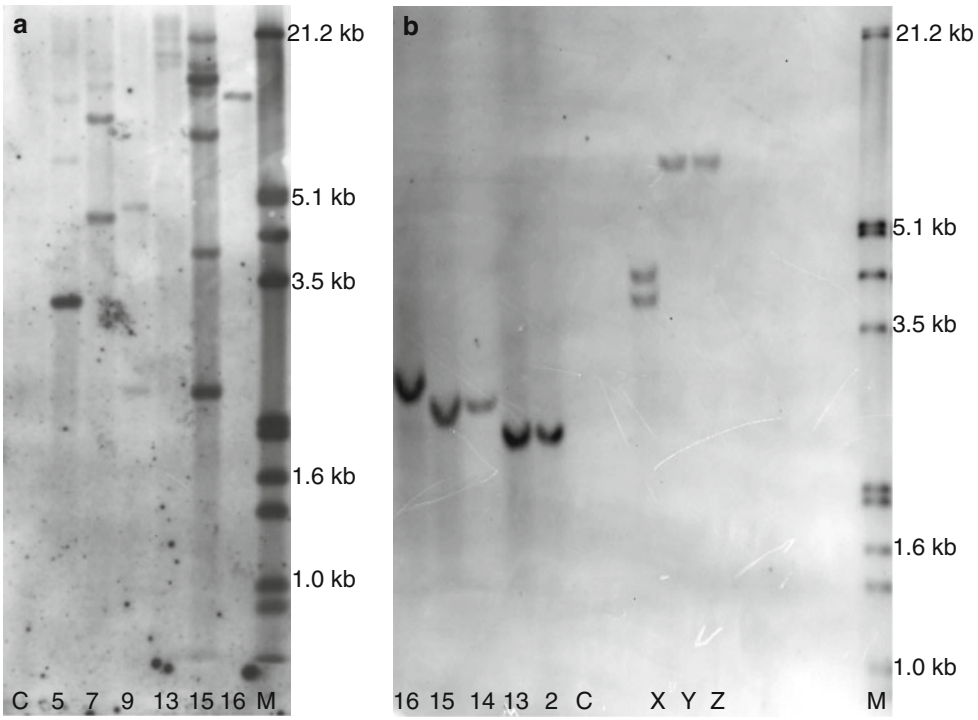


Fig. 2 Southern blot autoradiogram of transgenic lines of *K. blossfeldiana* (**a**) and *P. hybrida* (**b**). The genomic DNA was digested with *Bam*HI (*Kalanchoë*) and *Hind*III (*Petunia*) and probed with a DIG-labelled probe that was formed using the NtGA_2ox-438 (*Kalanchoë)* and HptII (*Petunia*) primer pairs (Table 1). M—DIG-labelled DNA molecular weight marker III; C—non-transgenic control; X, Y, Z—transgenic *Petunia* plants not described in this paper

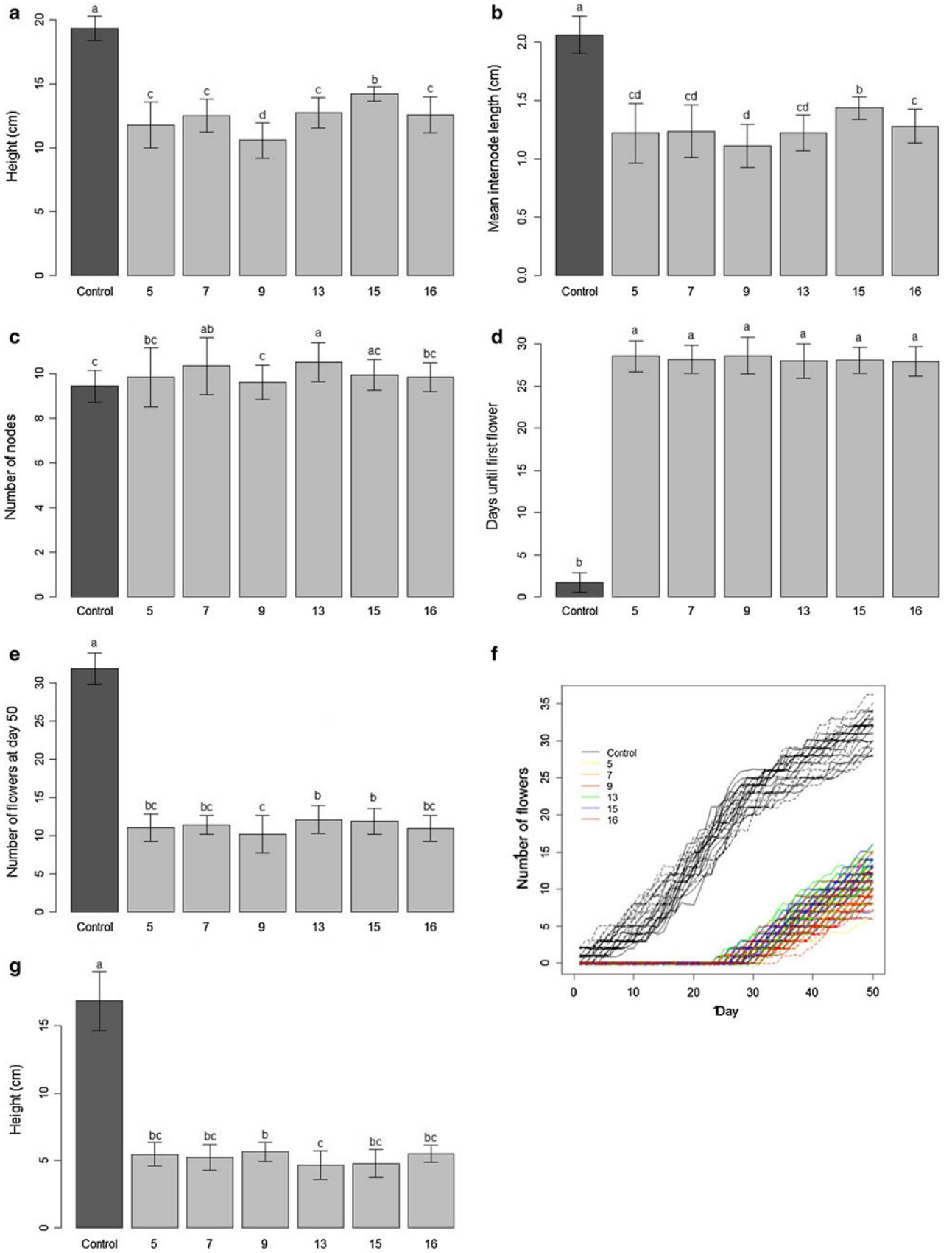
PCR, RT-PCR and Southern blot hybridisation

All investigated clones showed a positive PCR signal in reactions with the $NtGA_2ox$-571 and HptII primers (Table 1) and a negative result for the pCAMBIA primers (data not shown). Positive RT-PCR amplification results were produced when using $NtGA_2ox$-306 and KbPP2 primer pairs for *Kalanchoë* and the $NtGA_2ox$-306 and CYP primer pairs for *Petunia* transgenic lines demonstrating the expression of the inserted *N. tabacum* GA_2ox as well as of the *KbPP2* and *CYP* housekeeping genes (Fig. 1). Negative RT-PCR results were produced for the non-transgenic control plants and non-template control samples (Fig. 1).

Fig. 4 Vegetative traits of *K. blossfeldiana* control plants and ▶ *35s::gibberellin 2-oxidase* (GA_2ox) transgenic lines: **a** Mean plant length (cm); **b** mean internode length (cm); **c** mean number of nodes per plant; **d** mean number of days until first open flower (anthesis); **e** mean number of open flowers at the 50th day of measurement; **f** number of flowers over time; and **g** mean inflorescence length after 6 weeks of SD exposure for the *Kalanchoë* control and transgenic lines. Bars marked with letters (**a**, **b**, **c**, **d**, **e**) are significantly different at $P < 0.05$ by Tukey's multiple range test. *Bars* represent the mean ± SD (n = 20)

Southern blot hybridisation of *Kalanchoë* transgenic lines demonstrated the different number of T-DNA integration events in different *Kalanchoë* clones (Fig. 1a). Clones K5 and K7 had four copies of T-DNA, clone K9

Fig. 3 Representative *K. blossfeldiana* and *P. hybrida* flowering control and transgenic lines grown under greenhouse conditions: **a** *Kalanchoë*; **b** *Petunia*; **c** Flowers of the control and transgenic *Petunia* plants at the same stage of flowering


a
Height (cm)
Control
5
7
9
13
15
16
b
Mean internode length (cm)
c
Number of nodes
d
Days until first flower
e
Number of flowers at day 50
f
Number of flowers
Day
g
Height (cm)

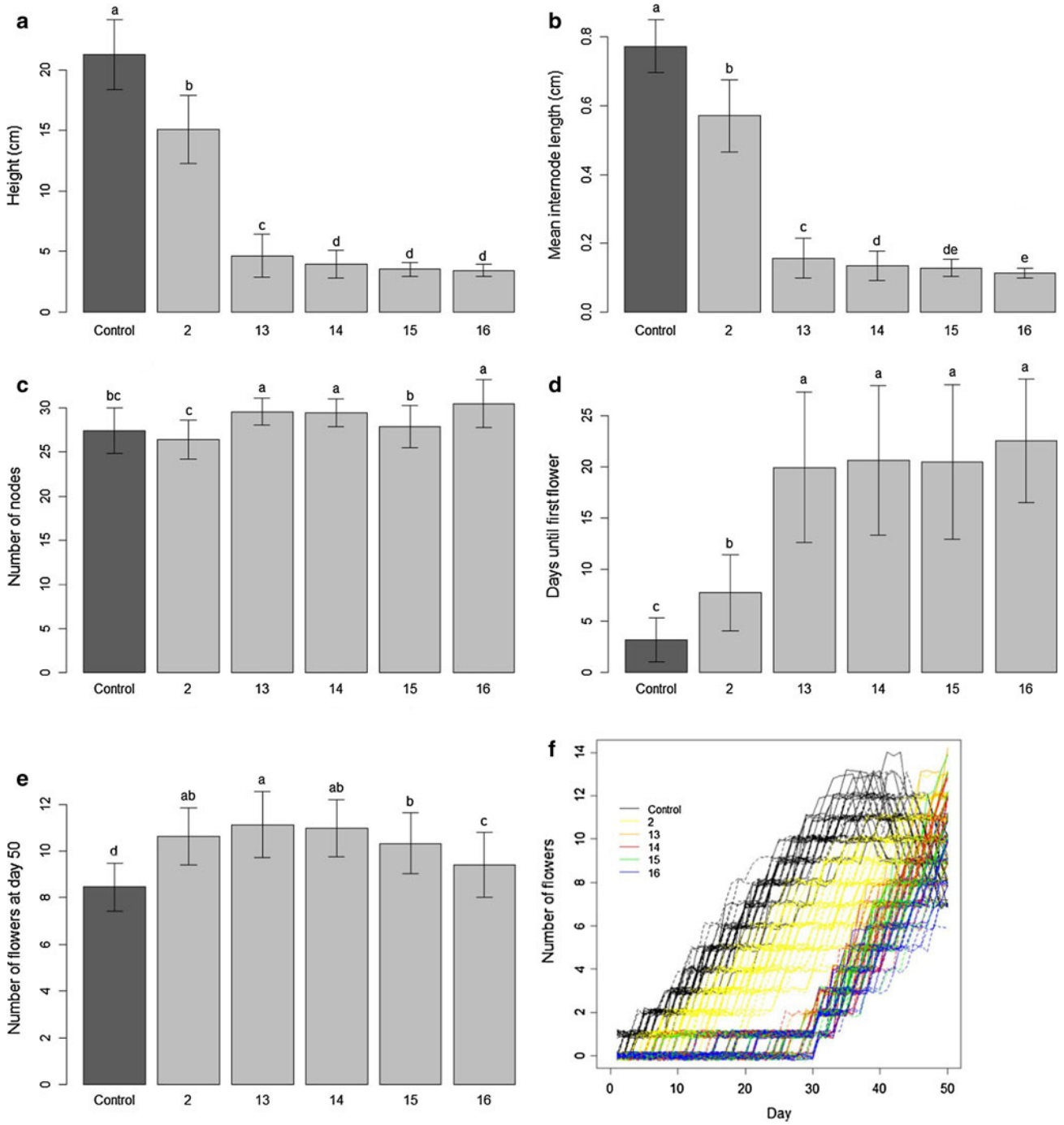


Fig. 5 Vegetative traits of the *P. hybrida* control plants and *35s::gibberellin 2-oxidase* (GA_2ox) transgenic lines: **a** Mean plant length (cm); **b** mean internode length (cm); **c** mean number of nodes per plant; **d** mean number of days until first open flower (anthesis); **e** mean number of open flowers at the 50th day of measurement; and **f** number of flowers over time. *Bars* marked with letters (*a*, *b*, *c*, *d*, *e*) are significantly different at $P < 0.05$ by Tukey's multiple range test. *Bars* represent the mean ± SD (n = 20)

had two copies, clone K13 had five, clone K15 had seven and clone K16 had one copy of the T-DNA. Although the number of T-DNA copies in the transgenic lines of *Kalanchoë* varied from one to seven, there were no large differences in the phenotypes between the different lines. In *Petunia*, all of the investigated transgenic lines possessed only one copy of T-DNA. There was, however, a significant difference in the growth and flowering habits between the *Petunia* line P2 and the other transgenic lines of *Petunia*, indicating a lower expression level of the transgene in P2 than in the other lines (Fig. 5). This effect could be caused by specific properties in the chromosomal

environments where the T-DNA integrated in P2. Perhaps the T-DNA integrated near a chromatin-controlling element or silencer, which could inhibit the expression of the transgene (Depicker and Van Montagu 1997; Frizzi and Huang 2010). It has also been reported that single-copy transgenes driven by the 35S promoter are more predisposed to gene silencing than transgenes driven by other promoters (Elmayan and Vaucheret 1996). The strength of the promoter has been correlated with the silencing frequency. The strength of the 35S promoter, in general, leads to high transgene expression levels, and it has been suggested that if the RNA level rises above a certain threshold, RNA degradation may occur (Que et al. 1997; Vaucheret et al. 1998; Marjanac et al. 2009). Other possible explanations for the *Petunia* P2 line's phenotypic characteristics may be somaclonal variation, DNA-based methylation or spontaneous mutations that might have occurred during regeneration under in vitro conditions in the transgenic plants (Cassells and Curry 2001).

Phenotypic evaluation of vegetative growth of transgenic lines

The lengths and mean internode lengths of all transgenic *Kalanchoë* and *Petunia* lines were significantly reduced in comparison to non-transgenic control plants (Figs. 4, 5). However, for transgenic clones of both species, the mean number of nodes was not changed significantly or was higher in comparison to the controls. The average length for the control plants of *Kalanchoë* was 19.3 cm with 9 nodes. The transgenic plants reached lengths between 10.5 (K9) and 14.5 cm (K15), and they possessed approximately 10 nodes on average. For the *Petunia* control plants, the average length of the stems was approximately 23 cm with 29 nodes. In line P2 (discussed in the previous section), the measured stem reached approximately 15 cm with 28 nodes on average. In the other transgenic lines, the lengths reached from 3.7 cm (P16) to approximately 5 cm (P13), and there were 32 nodes on average. All measurements were performed on the 28th day of observations. The magnitude of the transgene effects was larger on *Petunia* than on *Kalanchoë* (Figs. 4, 5).

Creating *Kalanchoë* plants with a compact growth habit has been the subject of several other studies. One approach was the transformation of the *Kb* 'Molly' cultivar with *rol*-genes (Christiansen et al. 2008). Another approach involved the down-regulation of $GA_{20}ox$ (involved in the synthesis of bioactive GAs) under the control of an ethanol-inducible promoter in the same cultivar (Topp Hovbye et al. 2008). A successful attempt at transforming several *Kalanchoë* cultivars with the *AtSHI* gene under a constitutive promoter has also been reported (Lütken et al. 2010). In these projects, a reduction in length was observed. Christiansen et al. (2008) reported that the mean internode length of the investigated plants was reduced, and the mean node number was higher in comparison with that of the control plants, in accordance with the results in the present work.

Phenotypic evaluation of reproductive growth of transgenic lines

In the present study, there was a significant difference between the *Kalanchoë* transgenic lines and the control plants in terms of the time until anthesis. Several clones of the transgenic *Kalanchoë* lines (K9, K13 and K16) reached anthesis of the first flower 24 days after the anthesis of the first flower in the control plants (Fig. 4f). At that time, the control plants already had 14 to 21 open flowers in the inflorescence. Several transgenic clones of *Petunia* from line P2 reached anthesis of the first flower 3 days after anthesis of the first flower in the control plants. Several *Petunia* lines reached anthesis 7 days after the control plants, and clones of the P16 line reached anthesis 12 days after the control plants. There were no differences in the morphology of the fully developed flowers between the control plants and transgenic lines, in the case of either *Kalanchoë* or *Petunia* (Fig. 3), with respect to the inflorescence per plant at the 50th day of observation. At observation day 50, the control *Kalanchoë* plants had 28 to 34 open flowers and the transgenic lines of *Kalanchoë* had six to 16 open flowers in the inflorescence per plant. The maximum number (22 to 32) of open flowers per inflorescence in the transgenic lines was reached at 63 to 71 days after the anthesis of the first flower. The maximum number of flowers per plant in the control *Petunia* plants occurred between 30 and 40 days after anthesis of the first flower. The number of flowers in the transgenic lines of *Petunia* ranged from six to 14 flowers per plant at observation day 50, and the number increased further. The average length of the inflorescence stems of the control *Kalanchoë* plants was approximately 17 cm at 6 weeks after the anthesis of the first open flower. The average length of the inflorescence stems of the *Kalanchoë* transgenic lines ranged from 4.6 cm for line K9 to 5.6 cm for line K13. The lengths of the inflorescence stems in the transgenic lines was significantly reduced in comparison to the inflorescence stems of the control plants.

The overexpression of GA_2ox, derived from *Phaseolus coccineus* under a constitutive promoter in *S. melanocerasum*, resulted in dwarfed plants for which, in contrast to the finding in the present work, the time of the first flowering in the transgenic and control plants was the same (Dijkstra et al. 2008). This result is the exception, because numerous studies show that mutant plants with reduced levels of active GA exhibit delayed flowering in addition to

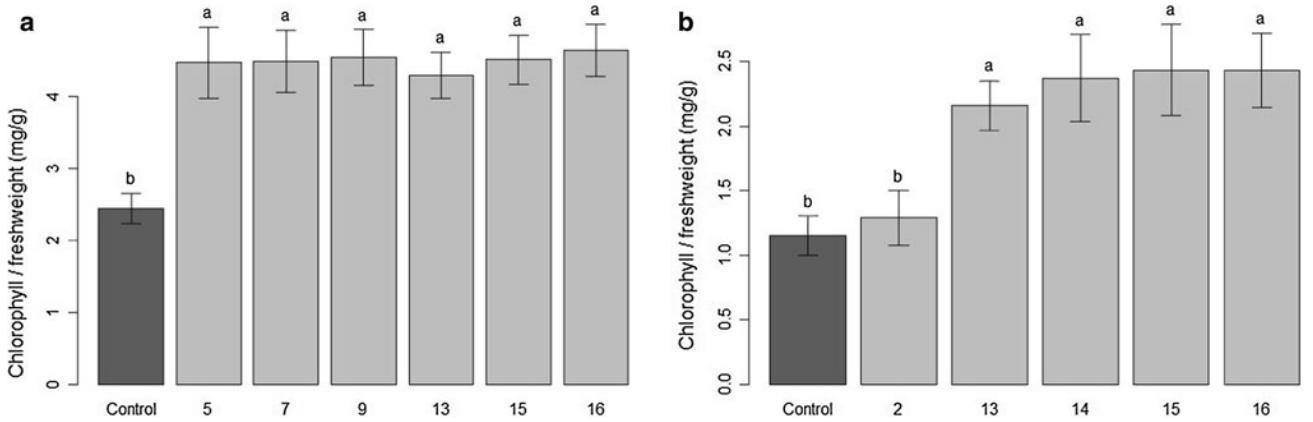


Fig. 6 Mean chlorophyll concentrations in the leaf tissues of **a** *K. blossfeldiana* and **b** *P. hybrida* in the control and transgenic lines, expressed as mg/(g fresh weight). *Bars* marked with letters (*a*, *b*) are significantly different at $P < 0.05$ by Tukey's multiple range test. *Bars* represent the mean ± SD (n = 20)

a dwarfed phenotype. It is believed that flower induction is controlled in different ways in different plant species. GAs promote flowering in some long day and biennial species through the activation of genes encoding floral pathway integrators, but their effects in other species is uncertain. In some perennials, GAs might inhibit flowering (Cleland and Zeevaart 1970; Hedden and Phillips 2000). Our study involves species with different flowering induction modes. *Kalanchoë* is a short day plant, and *Petunia* remains to be categorised. Nevertheless, the transgenic lines of both species exhibited significantly delayed flowering (Figs. 4, 5). Christiansen et al. (2008) reported similar results concerning the time to anthesis for *Kalanchoë* with inserted *rol* genes, and the same effect was observed in *Kalanchoë* lines with down-regulated $GA_{20}ox$ expression (Topp Hovbye et al. 2008). However, in several lines of *Kalanchoë*, different cultivars transformed with the *AtSHI* gene under the 35S promoter exhibited no significant delay in the first flowering after the start of flower induction in comparison to non-transgenic control plants (Lütken et al. 2010). Curtis et al. (2005) showed that among the *Arabidopsis* lines transformed with $PcGA_2ox_1$ under an estradiol-inducible promoter, the lines that exhibited the most severe dwarfism also had the longest delay in time to bolting and anthesis.

Chlorophyll content

The chlorophyll concentration in the leaves was almost doubled in the transgenic lines of both species compared with the control plants, with the exception of the *Petunia* line P2 (Fig. 6), which had almost the same chlorophyll concentration as the control plants. The transgenic lines of *Kalanchoë* plants had approximately 4 mg g^{-1} chlorophyll per fresh weight, while the control plants had only 2.5 mg g^{-1} on average. The transgenic lines of *Petunia*, except line P2, had over 2.2 mg g^{-1} chlorophyll per fresh weight, while the control plants and line P2 had slightly more than 1.0 mg g^{-1} chlorophyll per fresh weight on average. The chlorophyll content measurements were included in the research as a measurable parameter related to the visual impression (darker or lighter green color of the leaves). For ornamental plants, the leaf morphology and color are important. It has been observed in several studies that transgenic lines with an increased expression level of GA_2ox and, therefore, a lower level of physiologically active GA had visibly darker leaves (Biemelt et al. 2004; Ubeda-Tomas et al. 2006; Dijkstra et al. 2008). This phenomenon may conceivably be due to a smaller cell size in the transgenic plants, which is not accompanied by a reduction in the number of chloroplasts per cell. GAs promote plant cell elongation and division (Richards et al. 2001; Jupe et al. 1988; Keyes et al. 1990; de Souza and MacAdam 2001; Rood et al. 1990), possibly via the increased expression of the xyloglucan endotransglucosylase gene (XET) (Uozu et al. 2000). This enzyme is responsible for the cleavage and re-joining of the primary cell wall xyloglucans (hemicelluloses), which play a key role in regulating cell wall expansion (Jan et al. 2004; Bourquin et al. 2002). Based on these previous reports and our results on the chlorophyll concentration levels, it seems probable that the leaves of the transgenic plants in the present study have a greater cell density and reduced cell size but no reduction in the number of chloroplasts per cell.

In conclusion, the present study demonstrates that the constitutive expression of $NtGA_2ox$ in transgenic lines of *Kalanchoë* and *Petunia* results in phenotypic changes, such as reduced growth, dark green leaves, alterations in leaf morphology (smaller, thicker, dark green leaves) and delayed flowering, similar to those previously observed in other species with GA deficiencies. These results demonstrate that the overexpression of GA_2ox may be a useful method for obtaining the compact growth of *Kalanchoë*

and *Petunia* species without the use of chemical growth retardants. However, because delayed flowering and alterations of the leaf morphology are disadvantageous traits for ornamental plants, the effects of transformations using constructs in which GA_2ox is controlled by a stem-specific promoter will be investigated in future studies.

Acknowledgments The authors would like to thank Prof. em. Bjarne M. Stummann, the University of Copenhagen, Denmark, for his valuable advice and critical review of the manuscript. This project was supported by a PhD research grant from the German Academic Exchange Service (DAAD) (Ref.: 323, PKZ: A/06/07504), which is gratefully acknowledged.

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Research paper 2

Plant Biotechnology Journal

Plant Biotechnology Journal (2015) **13**, pp. 51–61 doi: 10.1111/pbi.12234

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

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Received 22 April 2014;
revised 17 June 2014;
accepted 26 June 2014.
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Accession numbers: *Kalanchoë blossfeldiana PP2*; KC782950.
Arabidopsis thaliana MKS1; AT1G21326.1.

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

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.

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_2ox) (Gargul *et al.*, 2013), transformation with *Agrobacterium rhizogenes* (Christiansen *et al.*, 2008), silencing of gibberellin 20 oxidase ($GA_{20}ox$) under an ethanol-inducible promoter (Topp Hovbye *et al.*, 2008) and overexpression of the short internodes gene (*SHI*) under the control of the 35S and *SHI* 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)
AtMKS1-570	*AtMKS1* cDNA in RT-PCR	Forward	CCAAAGACAACTGCAAACCA	570
		Reverse	TGCTCACCAAATCCAATCAA	
*Ph*VIGS-134	*MKS1* in *Petunia* cDNA	Forward	CCACTTCAGCAACTGCCTCGT	134
		Reverse	TCCTTCAGGGGTTCTTGTTTTCTC	
*Ph*VIGS-264	*MKS1* in *Petunia* cDNA	Forward	CGGAAAGTCACCGAGAAGAG	264
		Reverse	GCAGTTGCTGAAGTGGAACA	
KbPP2	*Kalanchoë protein phosphatase*	Forward	GGGGAAGTTTGCTGCTACTG	255
	2 gene	Reverse	GCAACCATGTAACGAACACG	
CYP	*Petunia cyclophilin* gene	Forward	AGGCTCATCATTCCACCGTGT	111
		Reverse	TCATCTGCGAACTTAGCACCG	

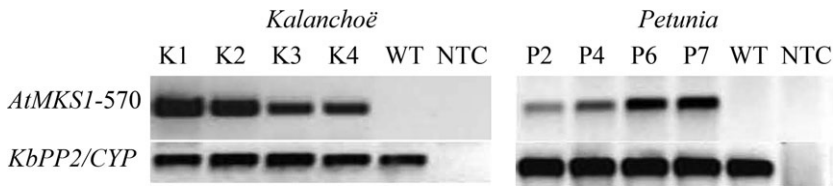

Figure 1 RT-PCR of transgenic *Kalanchoë* and *Petunia* lines. WT indicates wild-type nontransgenic control cDNA, and NTC is a no-template control. RT-PCR was performed using the *AtMKS1*-570 primer pair for both species. The *KbPP2* primer pair was used for *Kalanchoë,* and the *CYP* primer pair was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

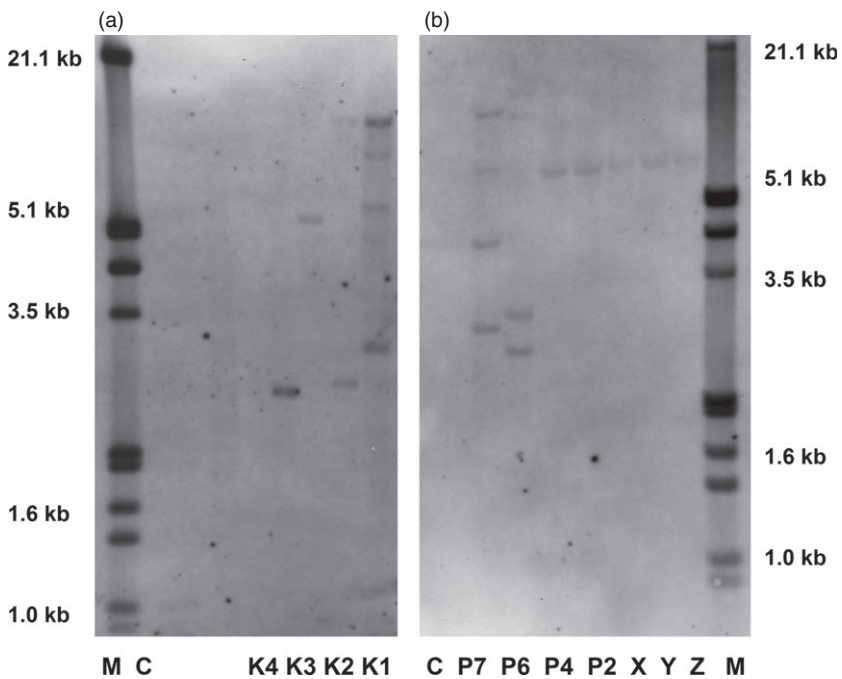

Figure 2 Southern blot autoradiogram of transgenic *Kalanchoë* (a) and *Petunia* (b) plants. Genomic DNA was digested with *Bam*HI (*Kalanchoë*) and *Hind*III (*Petunia*) and analysed using a DIG-labelled probe formed using the *AtMKS1*-570 primer pair (Table 1). M - DIG-labelled DNA molecular weight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

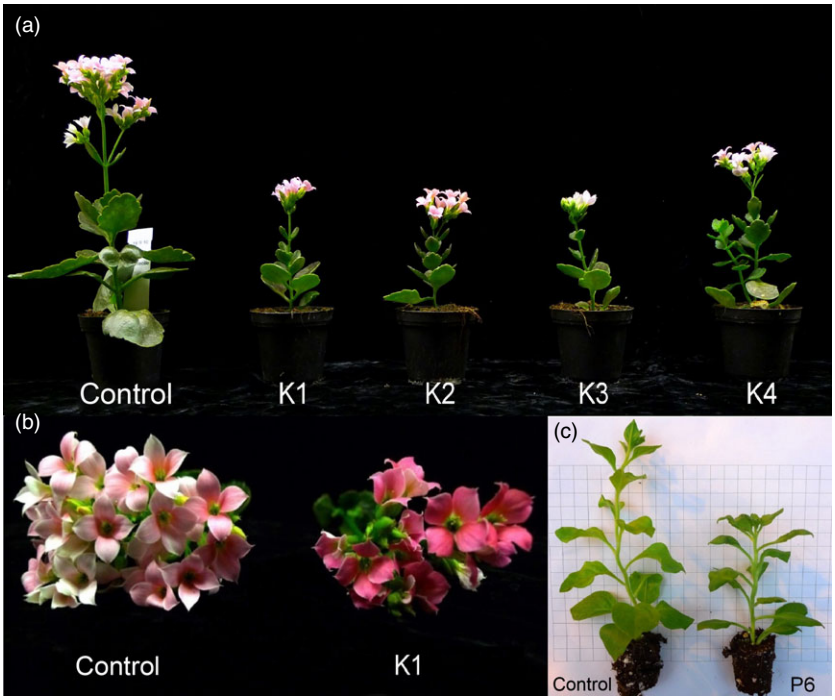

Figure 3 Control and transgenic lines grown under greenhouse conditions. (a) *Kalanchoë* plants (C – control); (b) flowers of the K1 line; (c) regenerated *Petunia* control and transgenic line P6 with the same number of nodes.

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 μM SA (optimum of 32 μM) 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.

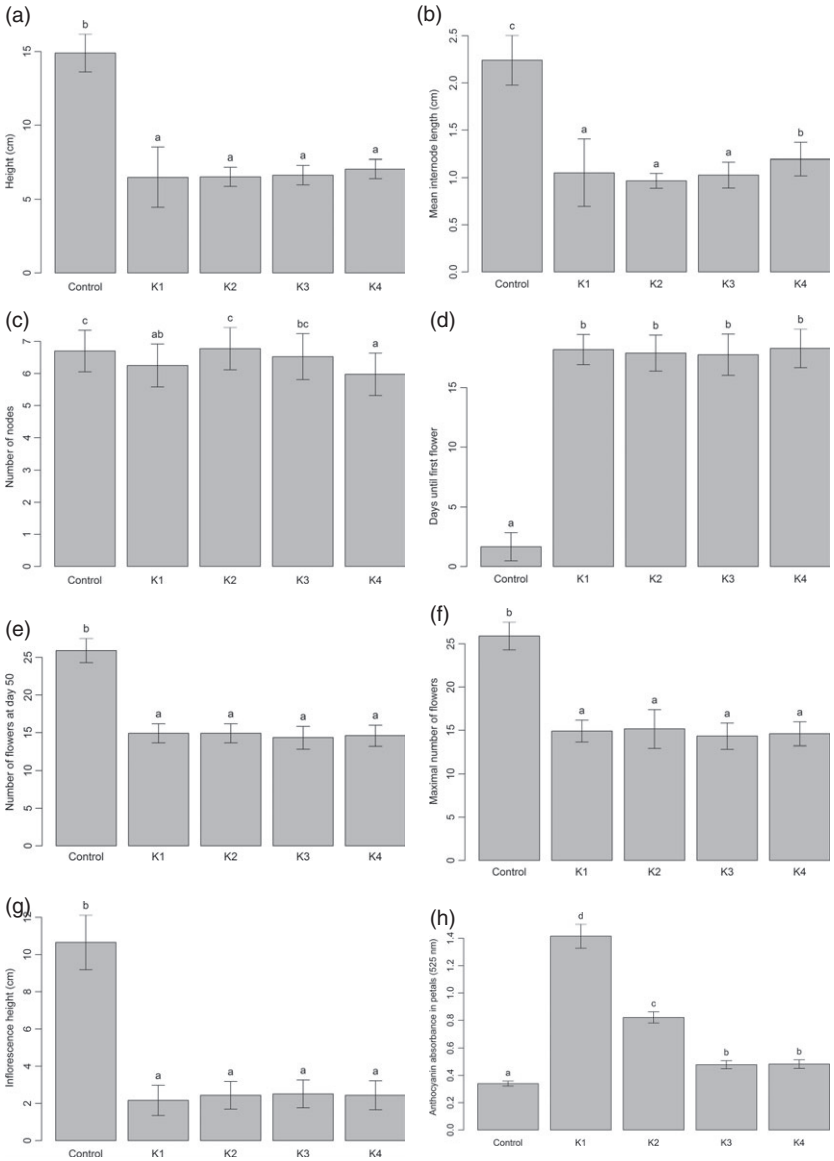

Figure 4 Phenotypic traits of *K. blossfeldiana* control plants and *35S::AtMKS1* transgenic lines. (a) Plant height (cm); (b) internode length (cm); (c) number of nodes per plant; (d) number of days until first open flower (anthesis); (e) number of open flowers at the 50th day of measurements; (f) maximal number of flowers per plant; (g) inflorescence height (cm) after 6 weeks under short-day conditions; and (h) anthocyanin absorbance at 520 nm (μmol/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 ± 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 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

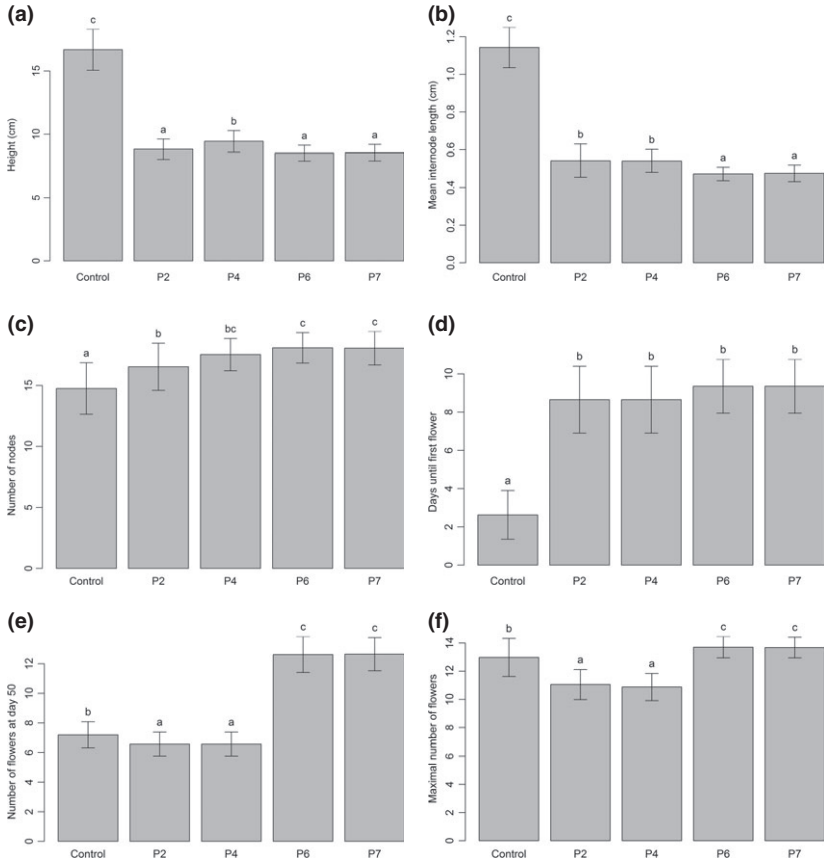

Figure 5 Phenotypic traits of *P. hybrida* control plants and *35S::AtMKS1* transgenic lines. (a) Plant height (cm); (b) internode length (cm); (c) number of nodes per plant; (d) number of days until first open flower (anthesis); (e) number of open flowers at the 50th day of measurements; and (f) maximal 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 ± 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

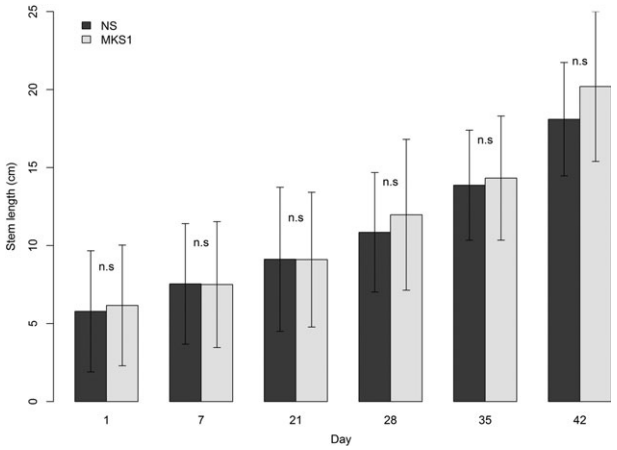

Figure 6 Comparison of the stem length (cm) of *Petunia* plants infiltrated with *PhMKS1*-VIGS and NS-VIGS (non-sense sequence, not the influencing phenotype) vectors. The length of the stems was measured weekly. The mean ± SD (n = 20) is shown.

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

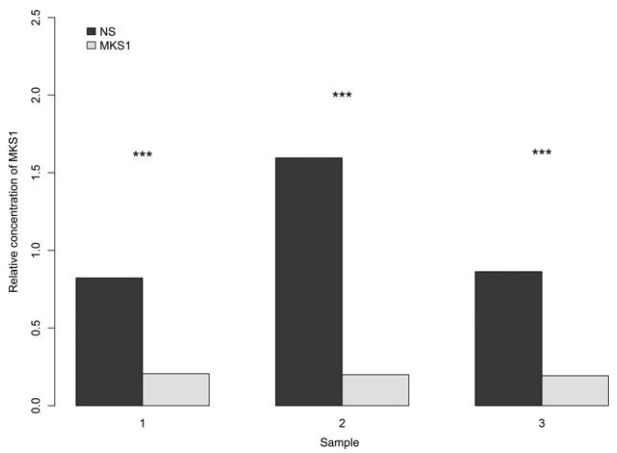

Figure 7 Comparison of relative *PhMKS1* gene expression levels in *Petunia* plants treated with *PhMKS1*-VIGS and NS-VIGS (qRT-PCR was repeated three times for three of the same *PhMKS*-VIGS-treated plants and three times for three the same NS-VIGS-treated plants). Significance code: ***P < 0.001 by 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).

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%

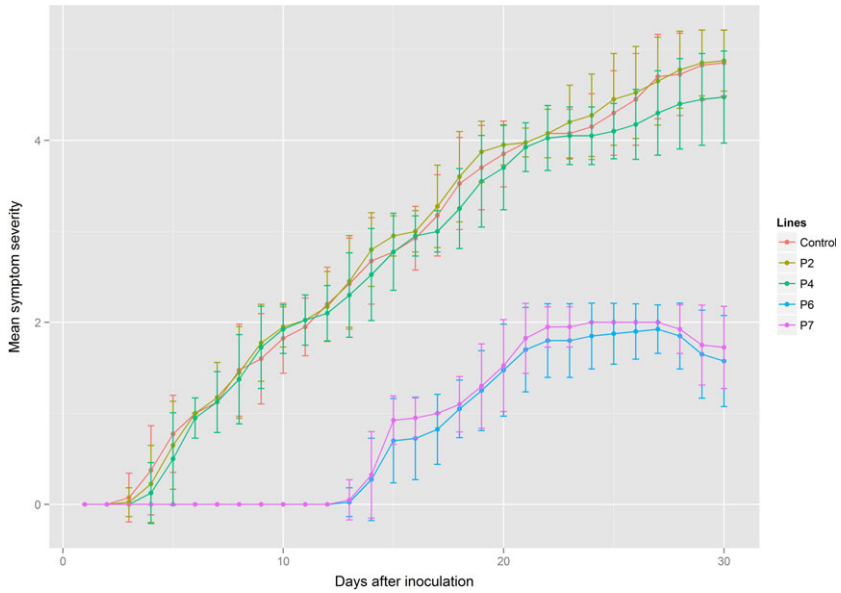

Figure 8 Severity of *Pseudomonas syringae* pv. *tomato* symptoms over time in *Petunia* control plants and transgenic lines P2, P4, P6 and P7. Observations were made daily for 30 days starting from infiltration. Symptom severity was ranked based on five stages, as described in the text.

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 *Ph*VIGS-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 *Eco*RI 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 $MgCl_2$ 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 photobleaching. 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 $MgCl_2$ 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).

Acknowledgements

The authors would like to thank Prof. John Mundy (University of Copenhagen, Denmark) for kindly providing the binary vector containing the *AtMKS1* sequence; Prof. Kerstin Wydra (Georg-August-University, Göttingen, Germany) for supplying the virulent strain of *P. syringae* pv. *tomato*; Dr. Merete Albrechtsen (University of Aarhus, Denmark) for providing the TRV vectors; and Prof. em. Bjarne M. Stummann (University of Copenhagen, Denmark) for his valuable advice and critical review of the manuscript. The authors would like to thank the staff of the floriculture section for technical assistance. This project was supported by a Ph.D. research grant from the German Academic Exchange Service (DAAD) (Ref.: 323, PKZ: A/06/07504), which is gratefully acknowledged.

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Scientia Horticulturae 197 (2015) 226–229

Contents lists available at ScienceDirect

Scientia Horticulturae

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Short communication

Phenotypic effects and the quantification of transcript abundance in *Petunia hybrida* 'Fantasy Blue' with virus-induced *GA$_2$ox* gene silencing

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ARTICLE INFO

Article history:
Received 2 March 2015
Received in revised form
19 September 2015
Accepted 25 September 2015
Available online 21 October 2015

Keywords:
qRT-PCR
Virus-induced gene silencing
VIGS
TRV
Gibberellin 2-oxidase

ABSTRACT

The aim of this study was to establish an efficient virus-induced gene silencing (VIGS) protocol for the gibberellin 2-oxidase gene (*GA$_2$ox*) in *Petunia hybrida* 'Fantasy Blue' to measure the reduction in GA$_2$ox transcript levels by quantitative RT-PCR (qRT-PCR) and to investigate the phenotypes of the infected plants. *In vitro* multiplied *Petunia* plants were subjected to VIGS using tobacco rattle virus vectors. An 838-bp fragment from the *N. tabacum* gene (*NtGA$_2$ox*) was cloned in the TRV2 vector. Control plants were also infected with a TRV2 vector containing a fragment of the *E. coli β-glucuronidase* (*GUS*) gene as a nonsense sequence. The abundance of the *GA$_2$ox* and *cyclophylin* (for normalization) transcripts was determined by qRT-PCR four weeks after inoculation. Shoot tips with the five youngest leaves were used. Stem lengths were measured weekly from the day of inoculation. After four weeks, significant increases in stem elongation were observed in *GA$_2$ox*-TRV2-infected plants compared with *GUS*-TRV2-infected plants. In accordance with this observation, the normalized abundance of the *GA$_2$ox* transcript in *GA$_2$ox*-TRV2-infected plants was significantly reduced compared with *GUS*-TRV2-infected plants.

1. Introduction

Virus-induced gene silencing (VIGS) is a method used for functional gene analysis (Lu et al., 2003; Chen et al., 2004; Purkayastha and Dasgupta, 2009). Several possible virus-derived vectors may be used for VIGS, but *Tobacco Rattle Virus*-derived vectors (TRVs) are generally useful in diverse plant species given the wide host range of TRV and minimal side effects in the treated plants (Ratcliff et al., 2001). TRV vectors silence gene expression in vegetative and floral meristems. Compared with vectors derived from other viruses, TRV vectors only induce mild disease symptoms. In addition, TRV vectors infect large areas of adjacent cells. TRV is two-partite virus with two separately encapsidated RNA genomes, RNA1 and RNA2. RNA1 encodes a movement protein, replicase proteins, and a cysteine-rich protein; RNA2 encodes two non-structural proteins and the coat protein. TRV RNA1 can replicate and move systemically in the plant in the absence of RNA2; thus, it is possible to substitute a portion of RNA2 with a sequence corresponding to genes targeted for silencing (Liu et al., 2002a; Senthil-Kumar and Mysore, 2014).

The TRV-based VIGS method uses binary *Agrobacterium tumefaciens* transformation vectors with T-DNA encoding TRV-RNA1 and TRV-RNA2 as well as 35S promoters for the transcription of viral sequences after the transfer of T-DNA to the plant cells. In the vector used, the sequence for the non-structural proteins in RNA2 is replaced with a multiple cloning site into which fragments of target genes can be inserted (Ratcliff et al., 2001,b; Liu et al., 2002a,b).

Petunia is one of the top-selling outdoor bedding plants worldwide with approximately 12 million plants sold in 2013 (Facts and figures 2013, Flora Holland, 2013). For comparison, sales of all other ornamental bedding plants consisted of 30 million pots in 2013 (Facts and figures 2013, Flora Holland, 2013). Various bedding plant species, including *Petunia* 'Fantasy Blue' or 'Picobella Blue', exhibit a compact growth habit; stem elongation may be beneficial for other commercial uses, including use in baskets or as ornamental ground cover plants. VIGS is a convenient method for rapid analysis of gene function through silencing and has been used to silence the *Petunia MKS1* gene (Gargul et al., 2015). This work describes the effect of *GA$_2$ox* VIGS on stem-length and other aspects of the phenotype of *Petunia hybrida* 'Fantasy Blue' as well as the associated reduction in *GA$_2$ox* transcript abundance as determined by qRT-PCR.

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http://dx.doi.org/10.1016/j.scienta.2015.09.039
0304-4238/

2. Materials and methods

2.1. Preparation of VIGS-vector constructs for plant infection.

A TRV-based vector system (Liu et al., 2002a; Ratcliff et al., 2001) was used to investigate the effect of *GA$_2$ox* gene silencing on the phenotype of *Petunia hybrida* 'Fantasy Blue'. A 1268-bp fragment of the *Nicotiana tabacum GA$_2$ox1* gene (acc. no.: AB125232.1) was amplified by PCR (forward primer, 5′CGGCAAATACTTGTCACTGAT3′; reverse primer, 5′TTTGCCTATGAACTTGTTTCATTAAC3′) and cloned into the T-easy vector (Promega Co., Madison, WI, USA) according to the manufacturer's protocol. The plasmid containing the *GA$_2$ox* fragment was digested with *Eco*RI (Thermo Scientific/Fermentas, Vilnius, Lithuania). The 838-bp fragment was purified by agarose gel electrophoresis and inserted into *Eco*RI-digested TRV2 vector. The ends of the *Eco*RI-digested TRV2 vector were dephosphorylated with FastAP^{TM} Thermosensitive Alkaline Phosphatase (Thermo Scientific/Fermentas) according to the manufacturer's protocol. Another TRV2 vector was prepared with a 798-bp fragment of the *β-glucuronidase* gene that was obtained from the pBI121 binary vector (acc. no. AF485783) by PCR amplification (forward primer, 5′TTTTTGTCACGCGCTATCAG3′; reverse primer, 5′CAACGAACTGAACTGGCAGA3′) to serve as a nonsense sequence (NS). As a control for the infection procedure, the *PDS* (*phytoene desaturase*) gene from *N. tabacum* was used as a reporter that causes leaf photo-bleaching (data not shown). All of the TRV2 constructs were assembled as described above and used to transform *Agrobacterium tumefaciens* strain GV3101. The bacterial cultures with TRV2 constructs and helper TRV1 constructs were prepared as described in Gargul et al. (2015). Plant infection was performed with harvested *Agrobacterium* cultures re-suspended in 10 mM $MgCl_2$ with 150 μM acetosyringone. Equal volumes of TRV1- and TRV2-becterial cultures were mixed and used for *Agro*-infiltration at 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.

2.2. Evaluation of phenotypes and the degree of gene silencing

Plants were maintained at 20 °C day/18 °C night temperatures and an average relative humidity of 70% under approximately 150 μmol m^{-2} s^{-1} of white lite with 16 h light/8 h dark cycles. Stem lengths were measured weekly after infection with *Agrobacterium*. After four weeks, infected tips, including the five latest leaves, from selected *Petunia* plants were picked for RNA isolation. RNA extraction and first strand cDNA synthesis was performed as described in Gargul et al. (2013). Quantitative RT-PCR mixtures were prepared at a final volume of 20 μL containing 0.5 ng of cDNA template, 0.25 μM forward primer and reverse primer, 2 U DCSHot DNA Polymerase (DNA Cloning Service, Hamburg), 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris HCl, 0.15 mM each dNTP (Jena Bioscience, Jena, Germany), and SYBR Green diluted 1:20,000 from the originally supplied stock solution SYBR Green I (Roche Applied Science Co. Mannheim, Germany). The following primers were used: 5′GAAGCCATCAAATTCTTCTCCTC3′ (forward) and 5′TTCGACCAAACCAACATCG3′ (reverse) for *GA$_2$ox* transcript; 5′AGGCTCATCATTCCACCGTGT3′ (forward) and 5′TCATCTGCGAACTTAGCACCG3′ (reverse) for the *CYP* (*cyclophylin*, Mallona et al., 2010) housekeeping reference gene transcript. The amplicon sizes were 117 bp for the *GA$_2$ox* primers and 111 bp for the *CYP* primers. To normalize the transcript levels, *CYP* and *GA$_2$ox* expression levels were detected concomitantly in the *GA$_2$ox*-TRV2- or NS-TRV2-treated samples using appropriate primer combinations. The DSCHot polymerase was thermally activated at 95 °C for 10 min to prevent nonspecific amplification, the extension of non-specifically annealed primers, and the formation of primer-dimers at low temperatures during PCR setup. Thermal activation was followed by 45 cycles of denaturation for 10 s at 94 °C, annealing for 30 s at 60–70 °C, and elongation for 30 s at 72 °C. Following the final PCR cycle, the specificity of PCR amplification was assessed by performing a melting curve analysis (from 68 to 95 °C). The PCR conditions were optimized for high amplification efficiency. Reactions were performed with the use of a Rotor Gene 3000 real-time thermal cycler (Corbett Life Science Co./Qiagen, Sydney, Australia), and data analysis was performed using the Rotor Gene software (6.1.81). The fold change in the *GA$_2$ox* transcript normalized to the *CYP* transcript between samples from *GA$_2$ox*-TRV2- and NS-TRV2-infected plants was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). A total of three independent repetitions of the qRT-PCR reactions were performed for three independent, randomly chosen plant samples. Each sample was represented by three technical replications (three reaction tubes) during the qRT-PCR procedure.

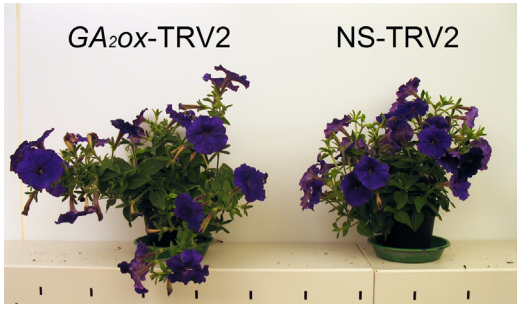

Fig. 1. Phenotypic comparison of *Petunia* plants with *GA$_2$ox*-TRV2 and NS-TRV2 (nonsense sequence) vectors at day 32 after infection. The stem length of *GA$_2$ox*-TRV2 treated plants was 13 cm and 22 cm in NS-TRV2 treated plants.

3. Results and discussion

The main stem lengths of the *Petunia* plants infected with *GA$_2$ox*-TRV2 and NS-TRV2 constructs clearly differed (Figs. 1 and 2). A statistically significant ($P < 0.05$) difference regarding main stem length, which averaged 1.6 cm, was observed on day 28 after infection. On day 42, the average difference had increased to 3.44 cm

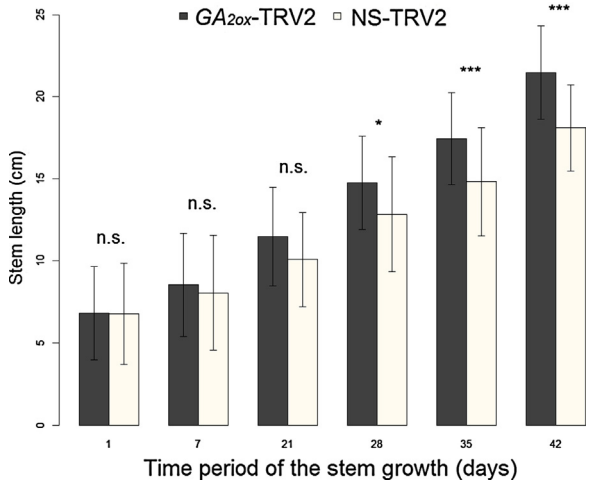

Fig. 2. Stem length comparison (cm) of *Petunia* plants infected with *GA$_2$ox*-TRV2 and NS-TRV2 vectors. Statistical analysis of stem length measurements in VIGS-treated plants was performed as previously described by Gargul et al. (2013); significance codes: '***', $P < 0.001$; '**', $P < 0.01$; '*', $P < 0.05$; '.', $P < 0.1$ and ' ' $P < 1$ based on log-transformation and a two-factorial analysis of variance. The length of the stems was measured weekly. Mean ± SD ($n = 20$) are shown.

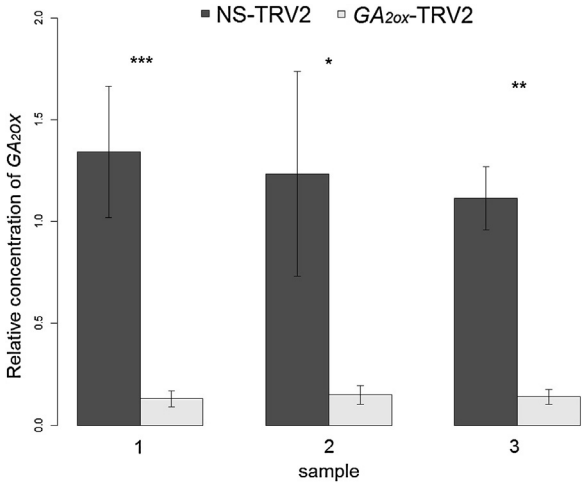

Fig. 3. Comparison of relative transcript abundance of *GA$_2$ox* genes in *Petunia* plants treated with *GA$_2$ox*-TRV2 and NS-TRV2 vectors; the qRT-PCR experiment was repeated thrice on each of 3 *GA$_2$ox*-TRV2-infected plants and 3 NS-TRV2-infected plants. The fold change in *GA$_2$ox* expression was calculated relative to the untreated control samples after normalization to the *CYP* gene. The expression levels in the untreated samples are set to 1 ($n = 3$ in all experiments). Means ± SD ($n = 3$) are presented. The statistical analysis and significance codes are the same as described in Fig. 2. The statistical calculations for the relative gene expression levels were performed as described by Gargul et al. (2015).

($P < 0.001$). To minimize the negative effects on phenotype, such as chlorosis, necrosis, lesions, or stunting, observed when using an empty pTRV2 vector as control (Hartl et al., 2008), a *GUS* sequence was introduced into a TRV2 vector as a nonsense, non-plant DNA control. The insert size in the in the TRV2 vector influences the phenotype (Broderick and Jones, 2014; Wu et al., 2011). *Petunia* and tomato plants inoculated with vectors containing inserts with fragments shorter than 200 bp exhibited TRV2 viral-derived lesions and necrosis, whereas 265-bp and 365-bp inserts in *Petunia* and an approximately 400-bp insert in tomato exhibited no significant virus-associated effects. In our investigations, the *GA$_2$ox* and *GUS* inserts were approximately 800 bp in length. It is therefore probable that the TRV2 inserts used did not possess strong viral effects other than the intended gene silencing in the treated plants. Consistent with this expectation, the infection did not produce significant effects in the nonsense-control plants (Fig. 1). The qRT-PCR results demonstrated significant differences in *GA$_2$ox* expression levels between *Petunia* plants infected with the *GA$_2$ox*-TRV2 and NS-TRV2 constructs (Fig. 3). In each of three independent qRT-PCR experiments (each performed thrice), relative *GA$_2$ox* expression was significantly reduced in *GA$_2$ox*-TRV2-infected plants (approximately 8 to 10 fold lower) compared with NS-TRV2-infected plants (first experiment $P < 0.001$, second $P < 0.05$, and third $P < 0.01$, cf. Fig. 3).

The increased stem length phenotype of treated *Petunia* plants results from of the down-regulation of the GA$_2$ox enzyme, which is responsible for deactivating GA$_1$, GA$_4$ and their precursors into inactive molecules via 2-β-hydroxylation (Hedden and Phillips, 2000). *GA$_2$ox* gene silencing causes an increase in the concentration of active gibberellin in plant tissues, which influences the elongation of plant internodes (Thomas et al., 1999). Three *GA$_2$ox* genes are present in *Petunia*, and the mRNA sequences of these genes are published in the NCBI (National Center for Biotechnology Information) database (*GA$_2$ox1*, acc. no. GU059939.2; *GA$_2$ox2*, JQ323102.1; *GA$_2$ox3*, JQ323101.1) (Altschul et al., 1997). All three genes are highly identical (85, 85, and 84%, respectively, based on coding sequence comparisons) to the coding sequence of the fragment used for the VIGS experiment, indicating that the phenotype may be attributed to the silencing of all three *Petunia GA$_2$ox* genes. The 3 *Petunia* coding sequences are 84–90% identical. Kokot (2012) described one additional *GA$_2$ox* gene from *Petunia hybrida* that is not listed in the NCBI database. The described sequence of *PhGA$_2$ox4* is 78% identical to the coding sequence of the insert used for the VIGS experiment (ClustalW2, EMBL, European Bioinformatics Institute, www.ebi.ac.uk/clustalW). *Nicotiana tabacum* and *Solanum lycopersicum* each have 5 *GA$_2$ox* genes. Thus, *Petunia* may conceivable possess one more *GA$_2$ox* gene in addition to the four described to date. However, results from the pRT-PCR evaluation of transcript abundance may still reflect the abundance of mRNAs from all of the *GA$_2$ox* gene homologs.

Using transgenic tobacco plants, Dayan et al. (2010) demonstrated that *GA$_2$ox* silencing is a more potent technique for inducing plant growth and fiber production than constitutive overexpression of *GA$_{20}$ox*. *GA$_2$ox* silencing significantly improved the plant growth characteristics, compared with wild type and GA$_{20}$-oxidase overexpressing plants.

Based on these investigations and the results of the present study, we conclude that *GA$_2$ox* silencing in bedding plants, such as *Petunia* varieties, may be beneficial for improving ornamental plant production when a method for stable gene down-regulation without systemic viral infection is implemented.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

The authors would like to thank Dr. Merete Albrechtsen (University of Aarhus, Denmark) for kindly providing the TRV vectors, and Prof. em. Bjarne M. Stummann (University of Copenhagen, Denmark) for his valuable advice and critical review of the manuscript. This project was supported by a Ph.D. research grant from the German Academic Exchange Service (DAAD) (Ref.: 323, PKZ: A/06/07504), which is gratefully acknowledged.

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J Plant Growth Regul
DOI 10.1007/s00344-016-9650-x

Characterization of Transgenic *Kalanchoë* and *Petunia* with Organ-Specific Expression of *GUS* or *GA_2ox* Genes Led by the Deletion BOX-I Version (*dBI*) of the *PAL1* Promoter

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Received: 2 May 2016 / Accepted: 28 September 2016

Abstract In the present work, transgenic *Kalanchoë blossfeldiana* and *Petunia hybrida* with overexpression of *Nicotiana GA_2ox* inserted in pCAMBIA1303 T-DNA are investigated. To avoid possible adverse effects of constitutive overexpression a modified *Pisum PAL1* promoter was used, in which the BOX-I AC-rich motif had been deleted (*dBI*—deletion BOX-I). To investigate the tissue-specificity of the *dBI* and *PAL1* promoters, their sequences were fused with the *GUS* gene, cloned into two types of vectors (pCAMBIA1303 and p6N) and introduced by *Agrobacterium*-mediated transformation into both species. GUS-transgenic lines were tested for the *GUS* mRNA in stems, leaves, and roots with the use of RT-PCR and GUS-stained tissues were visualized and compared with the use of light microscopy. The *dBI* promoter leads to expression of *GUS* mRNA and GUS activity in stems and petioles but not in roots or leaves, whereas the *PAL1* promoter is less specific. All transgenic lines were tested for transgene copy number using Southern blot analysis. Transgenic *Kalanchoë* plants with *Nicotiana GA_2ox* exhibited more than twofold reduction in stem length but no reduction of the number of internodes. Similarly, in *Petunia* transgenic plants, the stem length was reduced threefold. The leaves of the transgenic *Kalanchoë* plants were smaller, as convex as those of the youngest leaves of the non-transgenic control plants, and had reduced petiole length. The leaves from transgenic *Petunia* plants were similar in shape to those of the non-transgenic control plants but significantly smaller.

Keywords 35S promoter · Compact growth · *GA_2ox* · *Kalanchoë* · *Petunia* · Stem- and petiole-specific promoter

Introduction

One of the most important qualities of ornamental plants is their growth habit. For several plant species, for example, *Kalanchoë, Hibiscus, Hydrangea, Rosa, Pelargonium*, and *Petunia*, breeders, ornamental plant growers, and trading companies prefer a compact growth phenotype for potted plants. Such plant growth architecture can be achieved by manipulating light and temperature conditions during the production process; they can also be achieved, to some degree, by changes in irrigation, pruning, or nutrition, but are mostly achieved by the application of chemical growth retardants (Rademacher 2000). Because the growth retardants may be toxic and have negative effects on the environment, several groups have investigated transgenic approaches as an alternative. For several plant species, it has been reported that manipulation to obtain a deficiency in bioactive gibberellic acid (GA) leads to more compact plant growth, but also to small dark green leaves, defective or delayed flowering, tillering, reduced seed production, male sterility or prolonged germination dormancy (in *Kalanchoë blossfeldiana* and *Petunia hybrida*, Gargul and others 2013; in *Petunia hybrida*, Liang and others 2014; in *Lolium temulentum*, King and Evans 2003; in *Oryza sativa*; Wang and Li 2005; Fleet and Sun 2005; Tanimoto 2005; Lo and others 2008; Sakamoto and others 2004). Decreased levels of bioactive GA may be obtained

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Published online: 21 January 2017

Table 1 Primers used and sizes of the resulting amplicons

Primer name	Target sequence/use	Directionality	Sequence (5′–3′)	Amplicon size (bp)
PsPAL1	*PsPAL1* sequence amplification	Forward	AATGCGATAAATCCCTCACG	1145
		Reverse	GCACCACCTTGTTTGGTTCT	
SOE	Splicing by overlap extension reaction	Forward	AACTTGTCTTTACTCACATATCAC	–
		Reverse	ATATGTGAGTAAAGACAAGTTGC	
HptII	*HptII* in T-DNA in genomic DNA and also in pCAMBIA and p6 vector (probe synthesis)	Forward	GATGTTGGCGACCTCGTATT	579
		Reverse	GATGTAGGAGGGCGTGGAT	
GUS-255	GUS in cDNA	Forward	TAATGTTCTGCGACGCTCAC	255
		Reverse	CCAGCCATGCACACTGATAC	
KbPP2	*Kalanchoë protein phosphatase 2* gene	Forward	GGGGAAGTTTGCTGCTACTG	255
		Reverse	GCAACCATGTAACGAACACG	
CYP	*Petunia cyclophilin* gene	Forward	AGGCTCATCATTCCACCGTGT	111
		Reverse	TCATCTGCGAACTTAGCACCG	

CYP primers previously described by Mallona and others (2010), HptII and *KbPP2* primers previously described by Gargul and others (2013)

by GA_2ox (*gibberellin-2-oxidase*) gene overexpression. The GA_2ox gene encodes an enzyme responsible for deactivation of gibberellin active forms (that is, GA_3) or its precursor molecules (that is, GA_8). To regulate the level of GA, the GA_2ox gene may be driven by a constitutive promoter or by a stem-specific promoter.

Constitutive promoters are promoters that drive gene expression in most or all tissues at most or all times. Usually they display moderate expression in most tissues, but typically the expression is higher in vascular or meristematic tissues (Odell and others 1985; Benfey and others 1990a). They usually are derived from promoters of plant housekeeping genes or plant virus genes. In basic transgenic plant research, the most commonly used promoter is the cauliflower mosaic virus (CaMV) 35S promoter (Odell and others 1985, 1988). The 35S promoter contains multiple tissue-specific elements within its sequence; therefore, the total activity of the 35S promoter is relatively high in most tissues from many different plant species (Lam and others 1989). However, it has been shown by Yoo and others (2005) that for certain vectors an enhancer activity associated with 35S promoters in transformation marker genes may affect expression levels and tissue localization patterns of transgenes in *Arabidopsis*. In contrast, native plant constitutive promoters (for example, from actin, ubiquitin or tubulin genes) usually do not consist of tissue-specific fragments; instead, they are composed of fragments that exhibit a high efficiency of transcription factor recruitment in almost all tissues and throughout most of the life cycle of a plant (Odell and others 1985; Benfey and others 1990a).

In 2001, Imura and others showed that deletion of the BOX-I cis element of the phenylalanine ammonia-lyase 1

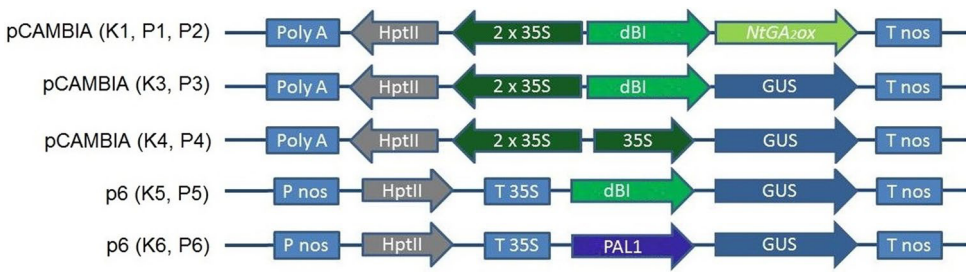

Fig. 1 Schemes of T-DNA constructs in the pCAMBIA1303 and p6N vectors used for transformation. In pCAMBIA, but not in p6N, the *HptII* gene used for selection of transformed plants is driven by a duplicated 35S promoter. Clones K1, P1 and P2 were transformed with pCAMBIA with the $NtGA_2ox$ gene driven by *dBI* promoter. K3 and P3 were transformed with pCAMBIA containing the *GUS* reporter gene driven by the *dBI* promoter. K4 and P4 were transformed with pCAMBIA containing the *GUS* gene driven by a 35S promoter. K5 and K6 were transformed with p6N with the *GUS* gene led by the *dBI* promoter. Finally, K6 and P6 were transformed with p6N with the *GUS* gene led by the *PAL1* promoter

Table 2 Length of stems and internodes of *dBI::GA$_2$ox*-transgenic lines after 35th days of observation

Control K	K1	Control P	P1	P2
Total stem length of the plant (cm)				
23.7 ± 1.33a	10.2 ± 1.15b	22.9 ± 3.7a	8.3 ± 1.06b	8.5 ± 0.84b
Length of the internodes (cm)				
2.35 ± 0.14a	0.9 ± 0.12b	0.82 ± 0.08a	0.25 ± 0.03c	0.28 ± 0.02b
Number of nodes				
10 ± 0.75b	11 ± 0.79a	27 ± 3.43b	32 ± 2.7a	29 ± 1.68b

In each row numbers labeled with different letters are significantly different at $P < 0.05$ by Tukey's multiple range test

The numbers are given as mean ± standard deviation ($n = 30$)

(*PAL1*) promoter sequence isolated from *Pisum sativum* leads to specific expression of the *GUS* reporter gene in xylem and phloem vessels of the *Nicotiana tabacum* stem. It was suggested that BOX-I in the *PsPAL1* promoter plays a role in negative regulation of expression in the stem but positive regulation in root and leaf tissues. The phenylalanine ammonia-lyase enzyme catalyzes the first step in the synthesis of phenylpropanoids, such as lignins impregnating xylem cell walls and suberins, and the constituents of the cell wall matrix in endodermal and phellogen tissues (Esau 1977).

In this study, we have modified the sequence of the *PAL1* promoter from *Pisum sativum* by deleting the BOX-I element, which resulted in a *dBI* promoter sequence. To compare the expression patterns and tissue specificity of regulation, the *dBI* and *PAL1* promoters were introduced in front of the *β-glucuronidase* (*GUS*) reporter gene. To illuminate the effect of 35S enhancer sequences (Yoo and others 2005), the *dBI* and *PAL1* promoters, and the *GUS* gene were cloned into two different vectors, pCAMBIA and p6N, which differ in the number of 35S promoter sequences in the T-DNA region. Finally, we constructed a pCAMBIA1303 vector with *GA$_2$ox* from *Nicotiana tabacum* driven by the *dBI* promoter inserted in place of one of the three 35S promoters in the T-DNA region. All constructed vectors were introduced into *Kalanchoë* and *Petunia* plants using *Agrobacterium*-mediated transformation.

Materials and Methods

Plant Material

Kalanchoë blossfeldiana '1998-469' and *Petunia hybrida* 'Famous Lilac Dark Vein' plants were introduced into and maintained in in vitro cultures as described in Gargul and others (2013). *Kalanchoë* plants were kindly provided by Knud Jepsen A/S (Hinnerup, Denmark), and *Petunia* plants were kindly provided by Selecta Klemm GmbH & Co. KG (Stuttgart, Germany). All experimental work was performed at the research facilities of the Section of Floriculture at Leibniz University of Hannover.

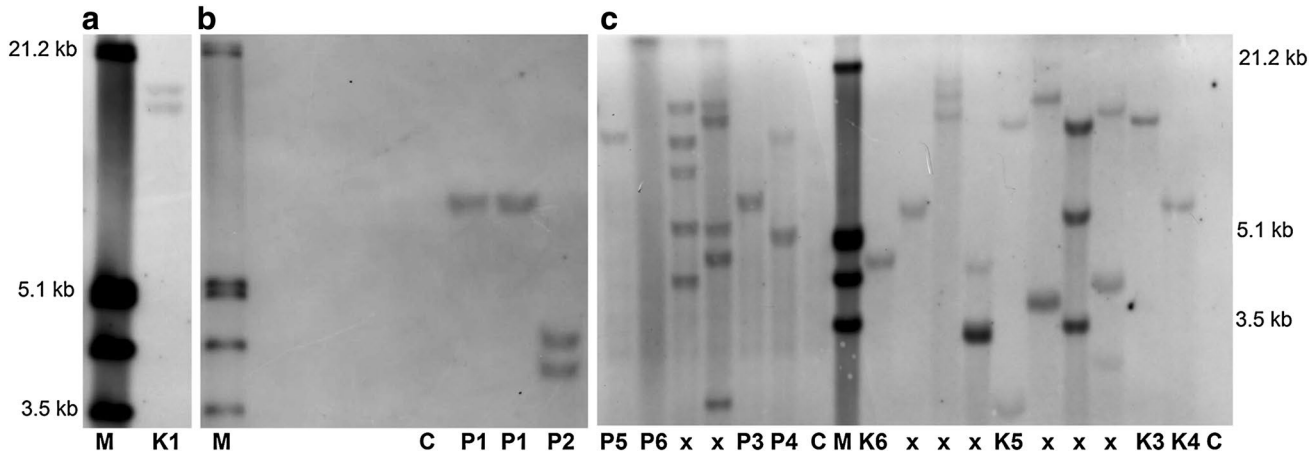

Fig. 2 Southern blots of transgenic K1 (**a**), P1, P2 (**b**) K3–K6 and P3–P6 (**c**) plants. Genomic DNA was digested with *Bam*HI (*Kalanchoë*) and *Hind*III (*Petunia*), and analyzed using a DIG-labeled probe formed using the *HptII* primer pair (Table 1). *M* DIG-labeled DNA molecular weight marker III; *C* nontransgenic control

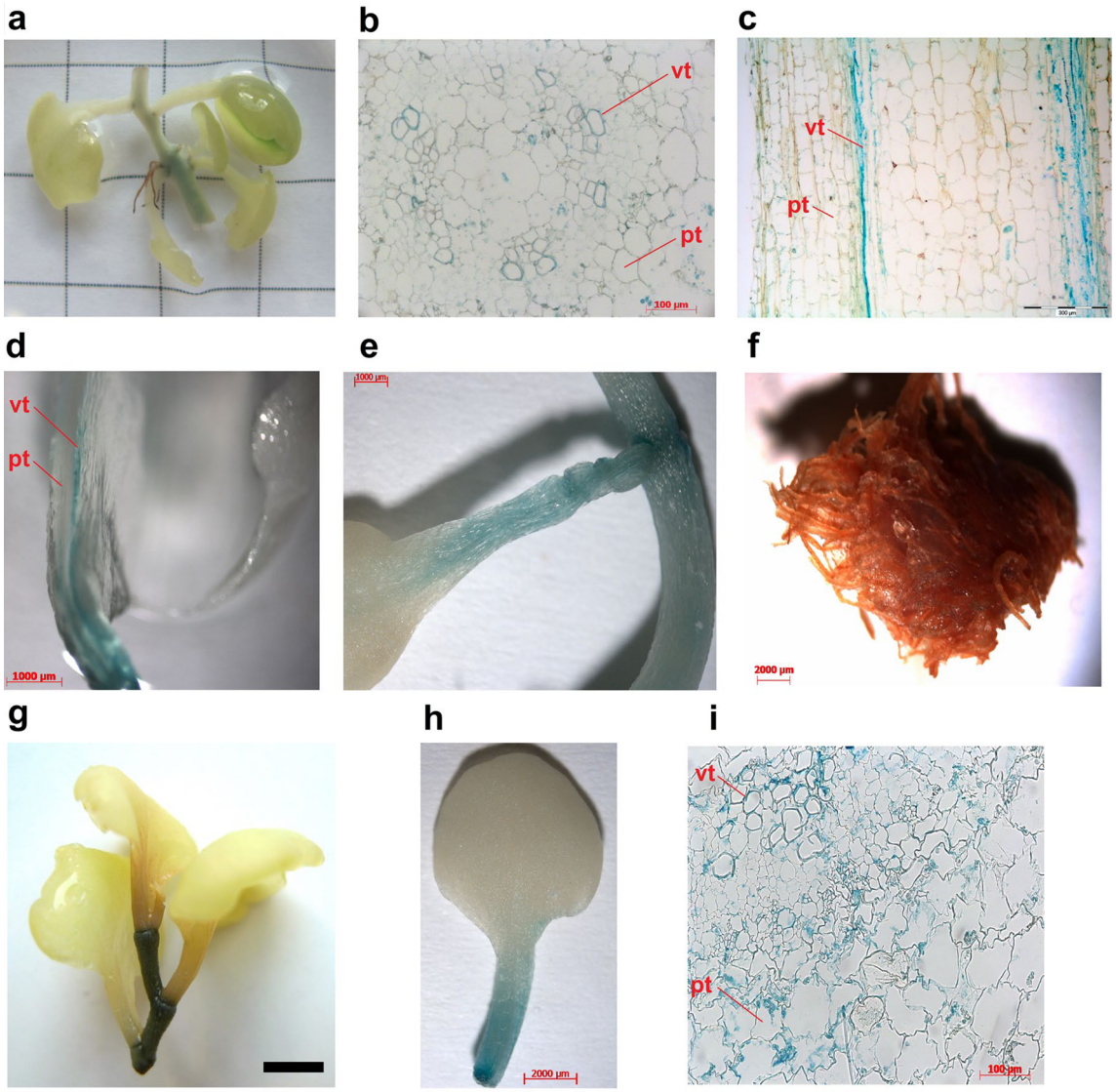

Fig. 3 GUS activity directed by the *PAL1* and *dBI* promoters in transgenic *Kalanchoë* plants. **a, d, e, f, o** K3; **b** K3—horizontal stem section; **c** K3—vertical stem section; **g, h, n** K5; **i** K6—horizontal stem section; **j, k, m** K6; **l** K6—vertical leaf section; **p** K5—horizontal stem cut; **q** K3—horizontal stem cut. *vt* vascular tissue, *pt* parenchymal tissue. In **a, j, k** each square represents 1 cm^2

Vector Construction

The promoter sequence of the *PAL1* gene (D10002.1) was amplified from *Pisum sativum* genomic DNA using the *PsPAL1* primer set resulting in a 1145 bp fragment that includes the first part of the transcribed sequence. The deletion of the cis-regulatory element BOX-I (13 bp) was performed using the splicing by overlap extension PCR method (Horton 1989) using both *PsPAL1* primers and SOE primers that were designed to overlap the deleted sequence (Table 1). The resultant mutated promoter is termed *dBI* for deletion BOX-I. The *dBI* promoter sequence was cloned into the pCAMBIA1303 vector in such a position that it replaced one of the 35S promoter sequence in front of the *β-glucuronidase* (*GUS*) gene. The *PAL1* and *dBI* promoters were also cloned into p6N vectors. The combination of $NtGA_2ox$ driven by the *dBI* promoter was constructed by replacing the 35S promoter with the *dBI* promoter sequence in a previously constructed pCAMBIA1303 vector containing $NtGA_2ox$ and described in Gargul and others (2013). A schematic view of the modified T-DNA constructs is shown in Fig. 1.

Plant Transformation

Plants of *Kalanchoë blossfeldiana* and *Petunia hybrida* were subjected to transformation with the *Agrobacterium tumefaciens* GV3101 strain following the protocol

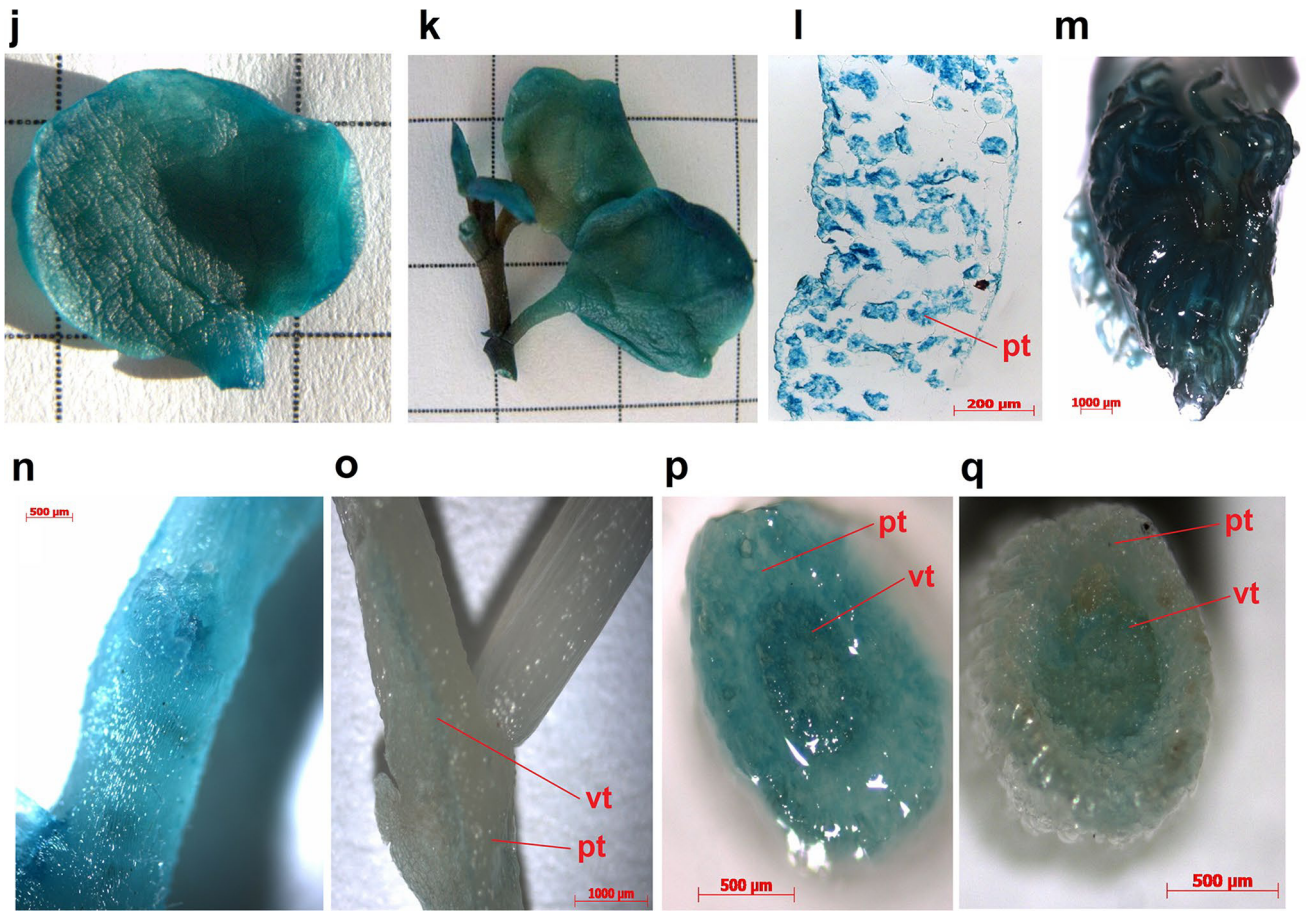

Fig. 3 (continued)

described by Gargul and others (2013). Plant regeneration, selection, and acclimatization of the transgenic lines were performed as described in Gargul and others (2013).

Southern Blot Hybridization

DNA of transgenic lines was isolated with the use of the Seqlab Kit according to the manufacturer's protocol and digested with *Bam*HI (*Kalanchoë*) and *Hind*III (*Petunia*) as described by Gargul and others (2013). Southern blots were performed as described by Sriskandarajah and others (2007). A digoxigenin-labeled probe targeting the *HptII* primer set was used according to the manufacturer's protocol (Roche Applied Science Co., Mannheim, Germany) (Table 1). Hybridization, post-hybridization and visualization of the hybridized fragments were performed as described by Sriskandarajah and others (2007).

GUS Histochemical Assay and Preparation of the Sections

Shoots of transgenic clones transformed with GUS-containing vectors were subjected to GUS histochemical staining with X-GlcA (Duchefa Biochemie B.V., Haarlem, The Netherlands) (Jefferson and others 1987). The solution consisted of 3 mM X-GlcA; 0.5 mM $NaPO_4$ (pH 7.0); 0.5 mM $K_4[Fe(CN)_6]$; 0.5 mM $K_3[Fe(CN)_6]$ and 0.5% Triton. *Kalanchoë* explants immersed in the X-GlcA solution were kept under vacuum conditions for 3 h to enable a thorough soaking of the tissue with the solution. *Petunia* explants were kept for 30 min under vacuum for thorough soaking of the tissue. The tubes with the explants were then incubated at 37 °C for approximately 20 h. Explants were later rinsed several times in 80% (v/v) ethanol until the chlorophyll was completely removed. Clear explants were embedded in paraplast, and sections of the embedded tissue were prepared as previously described in Jedrzejuk and others (2012). Observations were made with a bright field [Axioskop Zeiss HBO 50/AC, Axiovert S100 (Carl Zeiss, Gottingen,

Germany) and OLYMPUS BX41 (Olympus, Tokyo, Japan)] microscopes.

RNA Isolation and RT-PCR

For RT-PCR verification of *NtGA$_2$ox* and GUS expression, total RNA isolation from all transgenic lines was performed with the use of the Invisorb Spin Plant RNA Mini Kit (Invitek & Co./STRATEC Molecular, Birkenfeld, Germany) according to the manufacturer's protocol. RNA was isolated from roots, stems, and leaves with petiole. Evaluation of RNA quality and quantity, digestion of genomic DNA in RNA samples, and cDNA synthesis was done as described by Gargul and others (2013). RT-PCR reactions were performed with the use of GUS-255 primer sets appropriate to the integrated transgene (Table 1). All investigated cDNA samples were also tested against expression of reference genes with *KbPP2* (for *Kalanchoë*, Gargul and others 2013) and *CYP* (for *Petunia*, Mallona and others 2010) primer pairs. RT-PCR reactions were performed as described by Gargul and others (2013).

Phenotype Evaluation of *dBI::GA$_2$ox*-Transgenic Plants

For the phenotypic analysis of *dBI::GA$_2$ox*-transgenic clones (*Kalanchoë*: K1 and *Petunia*: P1, P2), all transgenic lines and WT plants were multiplied from tip cuttings with the same number of nodes. Measurements of the total stem length and number of nodes were done weekly for 5 weeks for both species. Cuttings of *Kalanchoë* were kept under long day conditions (16 h of light) and cuttings of *Petunia* were maintained in a greenhouse under short day conditions (8.5 h of light) at 22/18 °C (day/night). The statistical analysis of the phenotypes of the transgenic lines (Table 2) was performed as previously described by Gargul and others (2013).

The experiments comply with the human and animal rights, law of Germany and ethical standards of the university.

Results and Discussion

Determination of T-DNA Copy Number of Transgenic Lines

The T-DNA constructs in vectors used for transformation in the present study are shown schematically in Fig. 1, and the Southern blots for determination of T-DNA copy number are shown in Fig. 2. For the lines transformed with the pCAMBIA1303 unmodified vector, Southern blot hybridization of the *Kalanchoë* clone (K4) revealed one copy of the T-DNA integrated in the genome, and for *Petunia* (P4) there were 2 copies of the transgene (Fig. 2c). Both *Kalanchoë* (K3) and *Petunia* (line P3a) transformed with pCAMBIA with the *dBI*::GUS modified cassette had one T-DNA copy (Fig. 2c). Line P3, transformed with the same construct as K3 and P3a, was not included in Southern blot hybridization. For the lines transformed with *dBI*::GUS cassette in p6N vector, *Kalanchoë* (K5) had 2 copies, and *Petunia* (P5) had one T-DNA copy in the genome (Fig. 2c). Finally, for the lines transformed with *PAL1*::GUS cassette in p6N vector, *Kalanchoë* (K6), had one T-DNA copy, and *Petunia* (P6) also had one T-DNA copy (Fig. 2c). For the lines transformed with *dBI::GA$_2$ox* cassette in pCAMBIA1303 vector, *Kalanchoë* (K1) had 2 T-DNA copies (Fig. 2a), and *Petunia* had one copy in P1 and two in P2 (Fig. 2b). The difference in copy number between the P1 and P2 lines had no obvious impact on the phenotype.

Comparison of GUS Protein Expression Patterns and RT-PCR Amplification Signals in Plants Transformed with pCAMBIA and p6N Vectors

We investigated the expression of *GUS* controlled by a modified *Pisum PAL1* promoter in which the BOX-I AC-rich motif had been deleted (a *dBI* promoter) and the influence of the presence of 35S enhancers on the *GUS* gene in transgenic *Kalanchoë* and *Petunia* plants by comparing the results obtained by transformation with pCAMBIA and p6N *GUS* vectors. An important difference between the vectors is that in pCAMBIA T-DNA the *HptII* gene is controlled by two 35S promoter sequences, whereas the p6N vectors use a P *nos* promoter for the same purpose (Fig. 1). The 35S enhancers may activate transcription of adjacent genes upstream and downstream of the insertion site (Benfey and others 1989, 1990b; van der Fits and others 2001; Borevitz and others 2000; Tani and others 2004); this activation can work on genes up to 3.6 kb (tetrameric 35S enhancer in *Arabidopsis*, Hsing and others 2007) or 12.5 kb (octameric 35S enhancer in rice, Weigel and others 2000) away from the insertion site.

A 1145 bp sequence comprising the promoter sequence of the *PAL1* gene (D10002.1) from *Pisum sativum* was isolated, and to create the *dBI* promoter 13 bp constituting the regulatory element BOX-I were deleted (see Experimental Procedures). According to Imura and others (2001), use of the *dBI* promoter leads to specific *GUS* expression in xylem and phloem of the stem in transgenic *Nicotiana*. The deleted BOX-I is an AC-rich cis-regulatory element that may act as a negative regulator of xylem expression in stem but as a positive regulator in root and leaf tissue expression (Imura and others 2001). We cloned the *dBI* and *PAL1* promoters before a *GUS* gene in pCAMBIA1303 and p6N T-DNA (Fig. 1) and transformed *Kalanchoë* and *Petunia* plants with these constructs. The localization of GUS

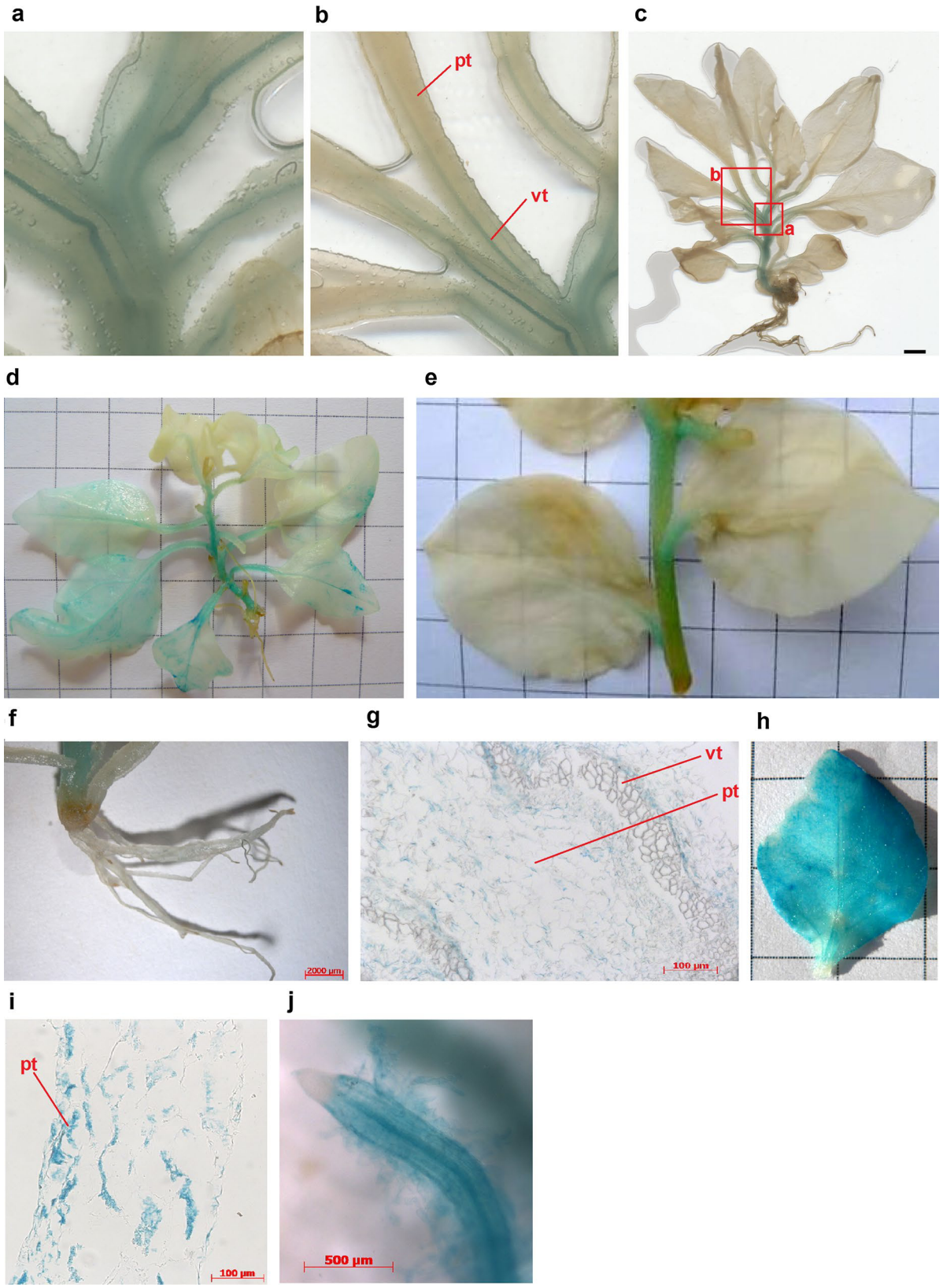

Fig. 4 GUS activity directed by the *PAL1* and *dBI* promoters in transgenic *Petunia* plants. **a–c** P3; **d** P3a; **e, f** P5; **g** P6—horizontal stem section; **j, h** P6; **i** P6—vertical leaf section. *vt* vascular tissue, *pt* parenchymal tissue. In **d, e, h** each square represents 1 cm^2

signals in the tissues subjected to the histochemical assay was observed and photographed (Figs. 3, 4).

The K3 and K5 transgenic *Kalanchoë* plants contain the *dBI* promoter before the *GUS* gene (compare Fig. 1). For both GUS protein expression is seen in stem and petiole (Fig. 3a–h, n–q). In contrast, for K6, which contains *PAL1*, expression is also seen in leaves and roots (Fig. 3i–m). Thus, deletion of BOX-I from *PAL1* makes the promoter stem and petiole specific in transgenic *Kalanchoë* in accordance with the results of Imura and others (2001). Because both K3 and K5 plants show stem and petiole specificity the two enhancers in pCAMBIA do not appear to have a negative effect in organ-specificity in transgenic *Kalanchoë*.

For *Petunia* P3 transgenic plants, which contain *dBI* promoter (cf. Fig. 1), GUS protein expression is seen in stem and petiole but not in leaf blades or roots (Fig. 4a–c). However, for the P3a clone GUS protein expression is not entirely specific, because an unexpected GUS signal localization pattern in leaves was revealed (Fig. 4d). For P5 the expression was specific for stem and petioles, because there were no signals in roots or leaf blades (Fig. 4e, f). For P6, which contains *PAL1*, there is, as expected, expression in stem (Fig. 4g), petiole, leaves (Fig. 4h, i), and roots (Fig. 4j). Thus, deletion of BOX-I from *PAL1* appears to make the promoter stem and petiole specific in *Kalanchoë* and also in *Petunia* transformed with p6N vector. The non-specificity of the GUS signals in the P3a plants might possibly be due to position effect, local chromatin structure, cytosine methylation, or the presence of T-DNA repeats at the site of the integration. All these effects have been previously reviewed by Matzke and Matzke (1998).

As mentioned above both K3 and K5 transgenic *Kalanchoë* plants, which contain the *dBI* promoter, show *GUS* expression in stem and petiole, but not in other examined organs. There is, however, a difference between the expression patterns in K3 and K5 (Fig. 3). For K5 no difference is seen between the GUS signal of vascular and parenchymal tissue of the stem and petiole (Fig. 3g, h, n, p), whereas the GUS signal for K3 is concentrated in the vascular tissue in accordance with Imura results (2001), (Fig. 3b–d, o, q).

All of the investigated *Kalanchoë* and *Petunia* lines showed positive RT-PCR amplification of mRNA from leaves, stems, and roots with primers targeted against the reference genes *KbPP2* (for *Kalanchoë*) (Acc. no.: KC782950) and *CYP* (for *Petunia*) (Table 1; Fig. 5). However, for RT-PCR amplification of mRNA from the *GUS* transgene with specific primers (Table 1), tissue differences were found for some of the investigated lines (Table 1; Fig. 5). In both species, the amplification was undetectable for root mRNA and strongly reduced for leaf mRNA for plants with the *dBI* promoter (K3, P3, K5, P5), except for the P3a line, where *GUS* mRNA was detected in leaves,

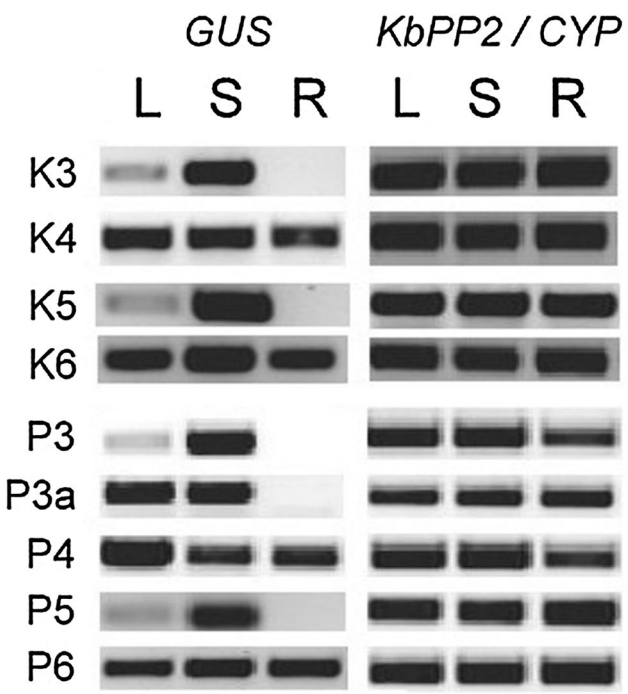

Fig. 5 RT-PCR of transgenic *Kalanchoë* and *Petunia* lines. RT-PCR was performed using the GUS-255 primer pair for both species. The *KbPP2* primer pair was used for *Kalanchoë*, and the *CYP* primer pair was used for *Petunia* (Table 1). Abbreviations signifying mRNA origin: *L* leaf, *S* stem, *R* roots

but not the roots (Fig. 5). For the lines with a 35S promoter (K4, P4) and *PAL1* promoter (K6, P6) mRNA was detected in all 3 tissues (Fig. 5). The mRNA expression pattern is consistent with the GUS protein expression pattern (Figs. 3, 4, 5).

A 35S enhancer influence on the transcriptional activities of the genes driven by the adjacent tissue-specific promoters has sometimes been detected (Zheng and others 2007; Singer and Cox 2013, Singer and others 2011, 2010a, b). It was first shown for a 35S enhancer located near nopaline synthase (nos), *gene 5* or *gene 7* promoters (Kay and others 1987; Odell and others 1988). Enhancers of 35S promoter appear to constitutively activate tissue-specific promoters, what results in erroneous expression in non-targeted tissues (Ren and others 2003; Weigel and others 2000). Comparison of the results for K3 and P3 (T-DNA with 2 35S sequences before the *HygR* gene, that is, near the *dBI* promoter), and K5 and P5 (T-DNA with no 35S sequence) may allow detection of possible effects of the presence of 35S enhancers on expression of GUS protein and *GUS* mRNA. No difference is seen in the RT-PCR results (Fig. 5). However, the GUS localization signals for K3 and P3 are found in the vascular tissues of stems (Figs. 3a–d, 4a–c) whereas the signals in stems are uniformly distributed for K5 and P5 (Figs. 3h, n, p, 4e). Thus, the expression of GUS protein, and therefore also of *GUS*

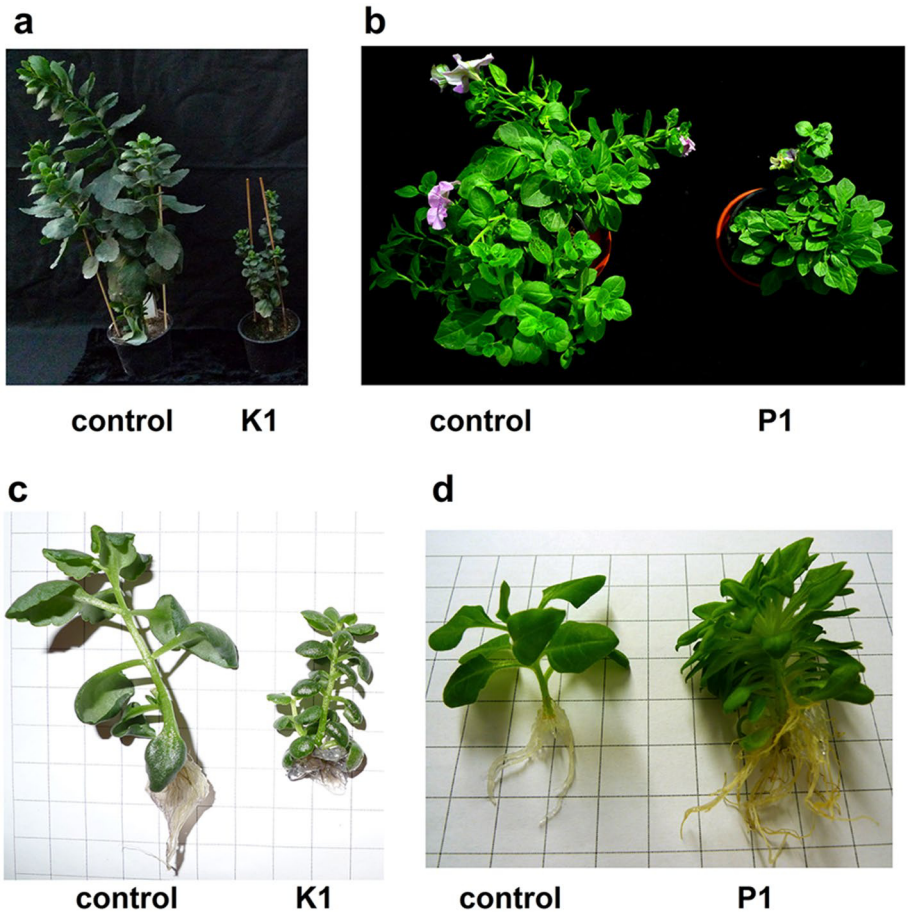

Fig. 6 **a, b** Control and transgenic lines grown under greenhouse conditions for 35 days. **c, d** Control and transgenic lines at the stage of root formation under in vitro conditions before acclimatization to greenhouse conditions. **a, c** *Kalanchoë* control and K1 plants. **b, d** *Petunia* control and P1 plants

mRNA, in stems is confined to vascular tissue in the presence of 35S enhancers near the *dBI* promoter. Both *Kalanchoë* and *Petunia* plants transformed with pCAMBIA1303 unmodified vector (K4, P4) exhibited uniformed GUS signal localization (not shown) and RT-PCR amplification signals (Fig. 5).

Stem and Internode Lengths for *dBI::GA$_2$ox*-Transgenic *Kalanchoë* and *Petunia* Plants

The average stem length was approximately 10 cm for the transgenic *Kalanchoë* (K1) plants and approximately 24 cm for non-transgenic wild-type (WT) plants (Table 2). Thus, the transgenic lines showed a significant, more than twofold reduction in stem length. The average internode length of the transgenic *Kalanchoë* K1 plants was approximately 0.9 cm, whereas in WT plants, the average was approximately 2.3 cm at the end of the observations (Table 2). The observations by Gargul and others (2013) of *Kalanchoë* (K1) and *Petunia* (P1) plants transformed with pCAMBIA1303 vector containing the *GA$_2$ox* gene driven by the 35S promoter were conducted according to the same procedures and under the same conditions as used in the present study. Comparing our previous study with the observations presented here, we can conclude that overexpression of *GA$_2$ox* under the *dBI* promoter results in a more compact phenotype in transgenic plants (Fig. 6). In *Kalanchoë* plants with constitutive overexpression of *GA$_2$ox*, the average stem length was decreased 1.8-fold, and the internode length was reduced twofold (Gargul and others 2013), whereas in *Kalanchoë* plants with stem-specific overexpression of *GA$_2$ox*, the total stem length was decreased 2.3-fold and the internode length was decreased more than 2.5-fold on average on the 35th day of observation (Table 2). In the present study as well as in the ones described by Gargul and others (2013, 2015), we investigated the *Kalanchoë* '1998-469' cultivar. This cultivar was selected as a result of communication with the head of the breeding department of the Queen *Kalanchoë*, A/S (Hinnerup, Denmark), who indicated that the '1998-469' cultivar exhibits the most elongated growth habit among common cultivars. In accordance with this, a comparison of the growth habits among selected cultivars of *Kalanchoë blossfeldiana* by Mibus and others (2014) demonstrated that the '1998-469' cultivar produces the most elongated stem in vegetative and also generative growth. There have been several successful trials aiming to reduce growth in *Kalanchoë blossfeldiana* cultivars (Lütken and others 2010; Christiansen and

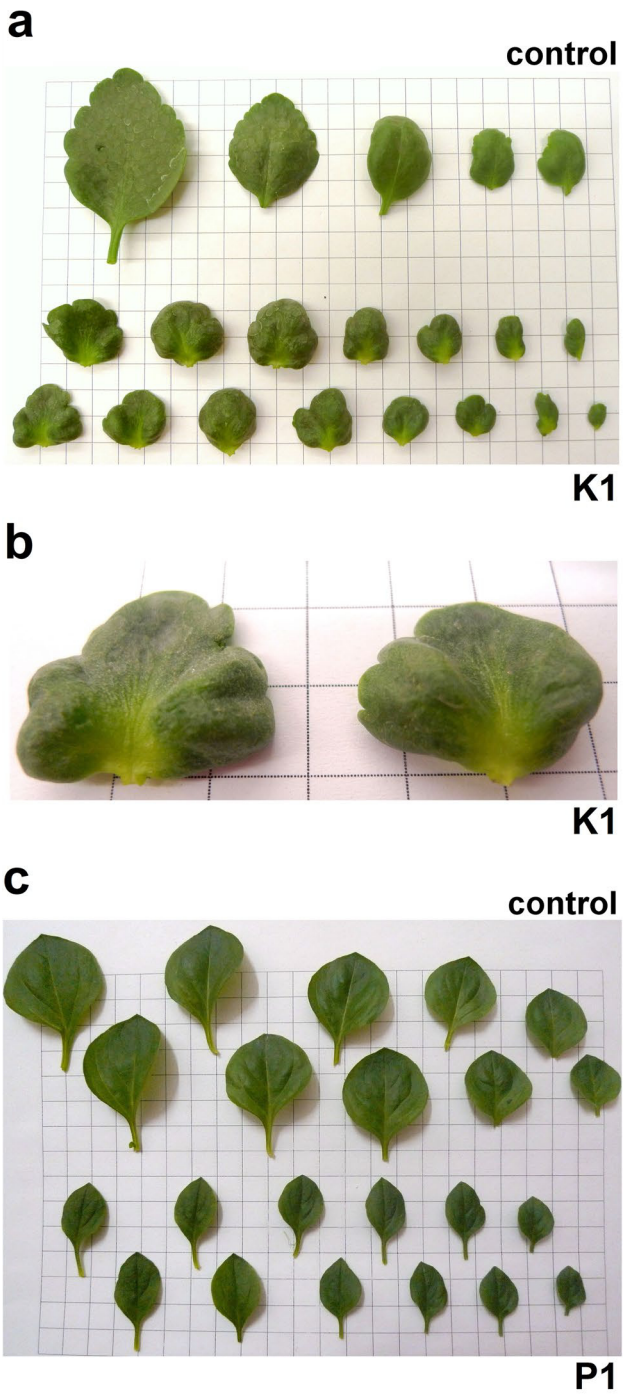

Fig. 7 Leaves from control and transgenic **a, b** *Kalanchoë* (K1) and **c** *Petunia* (P1) lines grown under greenhouse conditions for 35 days. Each square represents 1 cm^2

others 2008; Topp and others 2008), which included cultivars other than '1998-469', but it is difficult to objectively compare results on plant growth reduction between different *Kalanchoë blossfeldiana* cultivars because they exhibit significant differences in growth habit.

In *Petunia* transgenic plants with stem-specific overexpression of GA_2ox, the average stem length was approximately 8 cm on average, and the average internode length was approximately 0.3 cm on the 35th day of observation (Table 2). The stem length of the WT was approximately 23 cm and the internode length 0.9 cm on average. Thus, the average reduction of the stem is about 2.9-fold, and the reduction of the internode length about threefold (Table 2). The stem lengths of *Petunia* lines that constitutively overexpress GA_2ox were on average reduced fourfold, whereas the mean numbers of nodes were similar or higher than in the wild-type control plants (Gargul and others 2013). Manipulation of GA homeostasis in *Petunia* has also been described by Liang and others (2014) with a similar growth retardation effect. In that study, 2 different approaches were used: virus-induced gene silencing of *Petunia GID1* genes and overexpression of *Arabidopsis gai* gene under the control of a dexamethasone-inducible promoter. However, the growth retardation produced by the silencing of *GID1* genes in *Petunia* resulted in phenotypes of dark green leaves and delayed flowering. The induced *gai* expression resulted in dramatic growth retardation and smaller leaf size.

Leaf Shape of *dBI::GA_2ox- Kalanchoë* and *Petunia* Transgenic Plants

We have previously shown that constitutive overexpression of GA_2ox in *Kalanchoë* and *Petunia* does not change leaf or petiole morphology (Gargul and others 2013). The surface of the leaf blade of the transgenic plants of both species (K1 and P1) presented here was approximately twofold reduced in comparison to the size of the corresponding leaf blade in the WT plants (measured from the shoot top) (Fig. 7). The leaf shape of transgenic *Kalanchoë* (K1) was round, the petiole strongly reduced in comparison to those of the WT (Fig. 7a, b). That could conceivably be because GA_2ox is expressed in the vascular veins, but not the parenchyma of the leaf and petiole, similarly to the GUS expression patterns seen for K3 in Fig. 3d. It is possible that the growth of the vascular veins is reduced while the growth of parenchymal tissue is unaltered compared to the WT plants. In contrast, the shape of all the leaf blades of the *Petunia* P3 transgenic plants were not deformed compared to the WT plants (Fig. 7c).

Both *Kalanchoë* and *Petunia* plants transformed with pCAMBIA1303 unmodified vector (K4, P4) exhibited uniform GUS localized signals (not shown), that were not limited to vascular tissue in stem and petiole. That seems to be in accordance with the fact that constitutive overexpression of GA_2ox under 35S control does not change leaf or petiole morphology in these species (Gargul and others 2013).

Further Outlook

Based on the results presented here, we suggest that the combination of the *GA_2ox* 'dwarfing gene' with a stem-specific promoter might be an efficient alternative to the use of chemical growth retardants. However, because the leaf morphology changes when the *dBI* promoter is used it would be desirable to change this promoter, or find another useful stem-specific promoter, to avoid this phenomenon.

Acknowledgements This project was supported by a Ph.D. research grant from the German Academic Exchange Service (DAAD) (Ref.: 323, PKZ: A/06/07504), which is gratefully acknowledged. The authors are grateful for the possibility of the use of the microscopes at the Department of Ornamental Plants, Faculty of Horticulture and Landscape Architecture, Warsaw University of Life Sciences, Poland. The assistance of technical staff, Herbert Geyer, Wiltrud von Oertzen, and Annette Steding, is kindly acknowledged. The authors would like to thank Prof. em. Bjarne M. Stummann (University of Copenhagen, Denmark) for his valuable advice and critical review of the manuscript.

Compliance with Ethical Standards

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

Ethical Standards The experiments comply with the law of Germany and ethical standards of the university.

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Molecular Approach to Plant Growth Regulation by Constitutive Overexpression of *Nicotiana GA$_2$ox* in *Kalanchoë blossfeldiana*

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Keywords: *GA$_2$ox*, *gibberellin 2-oxidase*, compact growth, dwarf phenotype

Abstract

The development of an alternative method to chemical treatment for growth retardation in crop and ornamental plant production has become a top target in recent breeding programs. The present work describes the phenotypes of transgenic *Kalanchoë blossfeldiana* plants with overexpressed *gibberellin 2-oxidase* (*GA$_2$ox*) from *Nicotiana tabacum* under the 35S promoter. The height of the transgenic *Kalanchoë* lines was reduced by a factor of almost 2 in comparison to the control plants, while the number of nodes was similar between the transgenic and control plants. The height of the inflorescence stem of the transgenic lines was approximately three times reduced. However, the transgenic clones exhibited a delay in flowering that amounted to approximately 24 days. The flower morphology for all of the investigated transgenic lines was the same as that for the control plants. The transgenic lines had visibly darker leaves containing approximately two times as much chlorophyll as the leaves of the control plants. These results demonstrate that the overexpression of *GA$_2$ox* may become a useful method for obtaining compact growth of horticultural species without the use of chemical growth retardants.

INTRODUCTION

One of the possibilities of plant growth regulation is genetic manipulation of gibberellic acid (GA) metabolism. GA is a phytohormone that plays a crucial role in plant growth, tissue differentiation and development (Hedden and Phillips, 2000). GA-deficient mutants are usually shorter than the wild type. GA, which is involved in signal transduction pathways, can, together with light signals, influence the flowering induction process. GA 2-oxidases belong to the group of 2-oxoglutarate-dependent dioxygenases. They are responsible for the deactivation of GA_1, GA_4 and their precursors by hydroxylation into inactive molecules (Hedden and Phillips 2000). In accordance with this mechanism, it has been shown that the overexpression of the *GA$_2$ox* gene results in compact phenotypes in *Solanum melanocerasum* and *Solanum nigrum* (Dijkstra et al., 2008), *Nicotiana tabacum* (Ubeda-Tomas et al., 2007), *Nicotiana sylvestris* (Lee and Zeevaart, 2005), *Oryza sativa* (Sakai et al., 2003; Sakamoto et al., 2003) and *Arabidopsis thaliana* (Hedden and Phillips, 2000). Based on those findings it was decided to examine the effect of *GA$_2$ox* in *Kalanchoë blossfeldiana* (*Crasulaceae*). *Kb* is a very important indoor ornamental plant species, with a number of plants sold per year of approximately 77 million and a turnover of EUR 55 million in 2012 (Flora Holland, 2013). Unfortunately *Kalanchoë* produces elongated inflorescence stem, which is an unfavorable trait that decreases the ornamental value of the plant. Furthermore, different *Kalanchoë* species develop elongated internodes over time. To overcome these problems, different *Kalanchoë* cultivars are treated with different chemical growth retardants at least twice per vegetative season (research & production manager Kai Lønne Nielsen; Knud Jepsen A/S - pers. commun.). Chemical growth retardants are considered to be hazardous for human health and environment. Therefore creating transgenic plants with compact phenotype is one of the top targets of recent breeding programs for ornamental plants.

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Proc. I[st] IS on Ornamentals in Africa
Eds.: J.M. Wesonga and A.M. Opiyo
Acta Hort. 1077, ISHS 2015

MATERIALS AND METHODS

Plant Material

Plants of *Kalanchoë blossfeldiana* cultivar '1998-469' were provided by Knud Jepsen A/S (Hinnerup, Denmark). Leaf tissue was cultured and regenerated shoots were maintained in vitro as previously described by Ilczuk et al. (2009).

Vector Construction and Plant Transformation

The *GA_2ox* gene sequence of *Nicotiana tabacum* was cloned (AB125232.1.) in front of the 35S constitutive promoter within a pCAMBIA1303 vector (Cf. Web references) in place of the GFP-GUS gene fusion. The T-DNA contains a hygromycin gene under a duplicated 35S promoter. Plant transformation was performed as described in Gargul et al. (2013). It was established that the effective concentration level of hygromycin as a selection agent for shoot induction media was 5 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime. For root induction, the selection medium was supplemented with 8 mg L^{-1} hygromycin and 500 mg L^{-1} cefotaxime. Regenerated shoots were excised from the explants and transferred to root induction media supplemented with selection antibiotics. The shoots were transferred to fresh media every three weeks until they produced a well-developed rooting system. Then the plants were transferred to greenhouse conditions.

Southern Blot Hybridisation of the Transgenic Plants

Genomic DNA was isolated with a Seqlab Kit (Sequence Laboratories, Göttingen, Germany) according to the manufacturer's protocol. Approximately 12 μg of DNA from control and transgenic lines was used for Southern blot hybridization. DNA was digested using 30 units of *BamH* I (Thermo Scientific/Fermentas, Vilnius, Lithuania) for 24 h, with an additional 15 units for the next 24 h. DNA fragments were separated on agarose-gels and transferred to a membrane as described in Sriskandarajah et al. (2007). The probe was labeled with digoxigenin by PCR according to the manufacturer's protocol (Roche Applied Science Co. Mannheim, Germany) with the use of plasmid DNA with the inserted construct containing the *$NtGA_2ox$* primer pair (forward: 5' CCCCTTGTC CTGAGATTCAA 3', reverse: 5' TGAGGCTGCAATTTTCTCAA 3'). Hybridisation, post-hybridisation and size estimation of the visualized fragments were performed as described in Sriskandarajah et al. (2007).

Phenotype Evaluation

Plants with confirmed integration of T-DNA in the genome were multiplied as tip cuttings with the same number of internodes. While the cuttings developed a strong rooting system, plants were subjected to the measurements. The total height and number of internodes were measured under long day conditions (16 h of light) at 22/18°C (day/night). Flowering was induced by the transfer of the plants to short day conditions (8.5 h of light). Open flowers were counted daily. The inflorescence stem of *Kalanchoë* was measured once after six weeks from anthesis of the first flower.

Chlorophyll Determination

Chlorophyll measurements were made from three 8 mm-diameter discs excised from the center of the blade of the third leaf from the top of the plant. The chlorophyll content was analyzed according to Lichtenthaler (1987). Extraction was performed in 80% (v/v) ethanol at 75°C for 10 min. Absorption was measured using a SmartSpec™ 3000 Spectrophotometer (BioRad, CA, USA) at 647, 664 and 700 nm. Chlorophyll content was calculated according to the equation:

$$\text{Chlorophyll a+b (μg/ml in extract)} = 5.24 \times (A_{664} - A_{700}) + 22 \times (A_{647} - A_{700})$$

where A is the absorbance at 647, 664 or 700 nm. The results were expressed as mg of chlorophyll per g of fresh weight of leaf tissue.

Statistical Methods for Phenotype Evaluation of Transgenic Lines

The data were analyzed by linear mixed models with time, replication, clone, and clone-time interaction as fixed factors. The variables height and height/nodes ratio were log-transformed before the analysis. After fitting the model, multiple comparison procedures (Hothorn et al., 2008) were used to compare the mean of the clones to the mean of the control plants for each variable at each time point, pooled over the two replications. The statistical analysis was made with the R 2.12.1 program (R Development Core Team, 2010).

RESULTS AND DISCUSSION

Southern Blot Hybridisation

Southern blot hybridisation of *Kalanchoë* transgenic lines showed that different numbers of T-DNA integration events had occurred in the investigated *Kalanchoë* lines (Fig. 1). Clone number 1 had one copy of T-DNA, clone no. 2 had seven copies, clone number 3 had five and clone number 4 had two copies of the T-DNA. Despite that the number of T-DNA inserts in the transgenic lines varied, there were not large differences in the phenotypes between the different transgenic lines.

Phenotypic Evaluation of Vegetative Growth of Transgenic Lines

The lengths and mean internode lengths of all transgenic lines were significantly reduced in comparison to non-transgenic control plants (Fig. 2). The mean number of nodes was not changed significantly or was higher in comparison to the control plants. The transgenic plants reached lengths between 10.5 (clone no. 4) and 14.5 cm (clone no. 2), and they had approximately 10 nodes on average (Table 1). The average length for the control plants was 19.3 cm with 9 nodes. The measurements were performed on the day 28 of observations.

Obtaining *Kalanchoë* plants with a stable compact growth habit has been the subject of different studies. One of the studies involved down-regulation of $GA_{20}ox$ under control of an ethanol-inducible promoter in *Kb* 'Molly' (Topp et al., 2008). Transforming several *Kalanchoë* cultivars with the *AtSHI* gene, which is involved in the GA signal transduction, under a constitutive promoter resulted in compact phenotype (Lütken et al., 2010). Another investigation used transformation of the *Kb* 'Molly' with Ri-plasmids, where the TL-DNA contains *rol*-genes and the TR-DNA contains two ORFs called aux1 and aux2, which are involved in auxin biosynthesis (Christiansen et al., 2008). In these studies it was reported that the mean internode length of the investigated plants was reduced and that the mean node number was higher in comparison with that of the control plants.

Phenotypic Evaluation of Reproductive Growth of Transgenic Lines

A significant difference between the *Kalanchoë* transgenic lines and control plants in terms of time until anthesis was observed. Clones no. 1, 3 and 4 reached anthesis of the first flower 24 days after the anthesis of the first flower in the control plants. At that point in time of measurements, the control plants already had 14-21 open flowers in the inflorescence (Table 2). There were no differences in the morphology of the fully developed flowers between the control plants and transgenic lines. At day 50 of the observations, the control plants had 28-34 open flowers and the transgenic lines had 6-16 open flowers in the inflorescence per plant. The maximum number of open flowers per inflorescence varied between 22 and 32 in the transgenic lines and was reached at 63-71 days after the anthesis of the first flower. The average length of the inflorescence stems were measured six weeks after anthesis of the first open flower. The length of the inflorescence stem of control *Kalanchoë* plants was approximately 17 cm, while in transgenic lines ranged from 4.6 cm for line no. 4 to 5.6 cm for line no. 3 (Table 2). The lengths of the inflorescence stems in the transgenic lines were significantly reduced in comparison to the inflorescence stems of the control plants.

Various other studies have shown that plants with modified GA synthesis or signal transduction in addition to a dwarfed phenotype also present delayed flowering. Such an effect was, e.g, found for *Kalanchoë* lines with down-regulated *$GA_{20}ox$* expression (Topp et al., 2008). However, in several lines of *Kalanchoë,* different cultivars transformed with the *AtSHI* gene under the 35S promoter exhibited no significant delay in the first flowering after the start of flower induction in comparison to non-transgenic control plants (Lütken et al., 2010). In case of *Arabidopsis* transformed with *$PcGA_2ox_1$* under an estradiol-inducible promoter it was found that the lines, with the most severe dwarfism also had the longest delay in time of bolting and anthesis (Curtis et al., 2005).

Chlorophyll Content

The chlorophyll concentration in the leaves of the transgenic lines had approximately 4 mg g^{-1} chlorophyll per fresh weight, while the control plants had only 2.5 mg g^{-1} on average (Table 1).

For ornamental plants, the leaf morphology and color are important. It has been reported in several studies that mutant plants with an increased expression level of *GA_2ox* and, therefore, a lower level of physiologically active GA had visibly darker leaves (Biemelt et al., 2004; Ubeda-Tomas et al., 2006; Dijkstra et al., 2008). This effect may be due to a smaller cell size in the transgenic plants, which is not accompanied by a reduction in the number of chloroplasts per cell. The positive influence of active GA on the plant cell elongation and division has been described in several studies (Jupe et al., 1988; Rood et al., 1990; Keyes et al., 1990; Richards et al., 2001; de Souza and MacAdam, 2001).

The present work demonstrates that the constitutive expression of *$NtGA_2ox$* in transgenic lines of *Kalanchoë* results in phenotypic changes, such as reduced growth, dark green leaves, and delayed flowering, which are similar to those previously described in other species with GA deficiencies. These results suggest that the overexpression of *GA_2ox* may be a useful method for obtaining compact growth of *Kalanchoë* without the use of chemical growth retardants.

ACKNOWLEDGEMENTS

The authors would like to thank Prof. em. Bjarne M. Stummann, the University of Copenhagen, Denmark, for linguistic correction of the manuscript. The authors would like to thank the staff of the floriculture section for technical assistance. This project was supported by a PhD research grant from the German Academic Exchange Service (DAAD) (Ref.: 323, PKZ: A/06/07504), which is gratefully acknowledged.

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Tables

Table 1. Phenotypic evaluation of vegetative growth of control and transgenic *Kalanchoë blossfeldianal* lines (K1-K4). Numbers labeled with different letters are significantly different according to the statistical analysis.

Control	K1	K2	K3	K4
Total stem length of the plant (cm)				
19.3 a	10.5 d	12.7 c	14.2 b	12.5 c
Length of the internodes (cm)				
2.05 a	1.09 d	1.2 cd	1.43 b	1.27 c
Number of nodes				
9.4 c	9.6 c	10.5 a	9.9 ac	9.8 bc
Chlorophyll (mg) per gram of fresh weight				
2.44 b	4.54 a	4.28 a	4.5 a	4.63 a

Table 2. Phenotypic evaluation of reproductive growth of control and transgenic *Kalanchoë blossfeldianal* lines (K1-K4). Numbers labeled with different letters are significantly different according to the statistical analysis.

Control	K1	K2	K3	K4
Length of the inflorescence stem (cm)				
16.8 a	5.6 b	4.6 c	4.7 bc	5.5 bc
Time to the anthesis of the first flower (days)				
1 b	28 a	27 a	27 a	27 a
Number of flowers at 50^{th} day of the observations				
31 a	10 c	12 b	11 b	10 c

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Figures

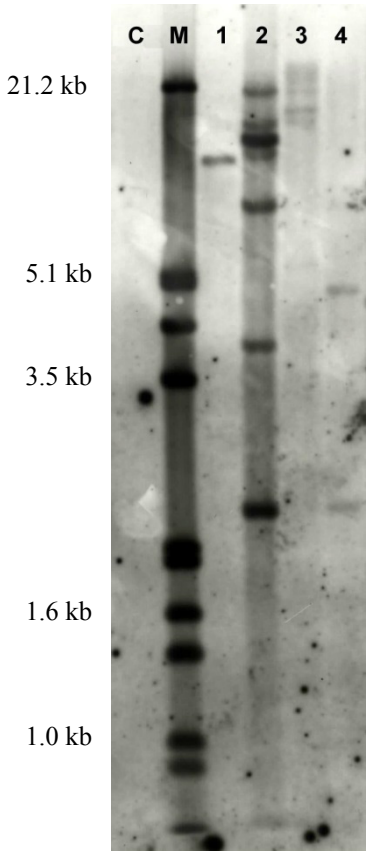

Fig. 1. Southern blot autoradiogram of transgenic lines of *Kalanchoë blossfeldiana*. The genomic DNA was digested with *BamHI* and probed with a DIG-labelled probe formed using the *NtGA$_2$ox* primer pairs. M - DIG-labelled DNA molecular weight marker III; C - non-transgenic control.

Fig. 2. Representative *Kalanchoë blossfeldiana* control and transgenic line grown under long day greenhouse conditions (16 h of light) at 22/18°C (day/night).

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Molecular Breeding of *Petunia hybrida* 'Famous Lilac Dark Vein' for Compact Growth Induction by *MKS1* and *GA$_2$ox* Constitutive Overexpression

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Keywords: compact growth, MAP kinase substrate 1, gibberellin 2-oxidase, growth retardation, dwarfed phenotype

Abstract

One of the top targets of recent breeding programs in ornamental plants is focused on developing an alternative way to the chemical growth retardants application in production of plants with compact growth habit. We describe work on *Petunia hybrida* 'Famous Lilac Dark Vein' plants with overexpressed *MAP kinase 4 nuclear substrate 1* (*MKS1*) and *gibberellin 2-oxidase* (*GA$_2$ox*) genes. *Petunia* plants were transformed with pCAMBIA modified vectors in separate experiments, containing constructs either with the *MKS1* or the *GA$_2$ox* gene under the control of *Ca35S* promoter. The transformation was performed with the use of *Agrobacterium tumefaciens* strain GV3101. The integration and expression of the transgene was investigated using Southern blot and RT-PCR analysis. The mean internode lengths of the *MKS1*-transgenic lines were 2.5-fold shorter than those of the wild-type plants. The flowering delay in *MKS1*-transgenic lines was observed with a range of 6 to 11 days in comparison to the wild-type plants. The mean internode lengths of the *GA$_2$ox*-transgenic lines were 6.5-fold shorter than those of the wild-type plants. The flowering delay in *GA$_2$ox*-transgenic lines was observed with a range of 7 to 12 days in comparison to the wild-type plants. The flower morphology either in *MKS1*- or *GA$_2$ox*-transgenic lines did not differ from wild-type plants. Constitutive overexpression of both *MKS1* and *GA$_2$ox* genes results in compact phenotype but also lead to delayed flowering.

INTRODUCTION

One of the aspects of molecular breeding of ornamental plant species is establishing a method of creating compact plants as an alternative approach to the utilization of chemical growth retardants. Here we describe the molecular breeding strategies based on the manipulation of *MKS1* and *GA$_2$ox* gene expression that influence the salicylic and gibberellic acid (SA and GA) phytohormonal balance. SA is involved mostly in plant growth, thermogenesis, flower induction, leaf abscission or ethylene biosynthesis. It has been reported that increase of SA concentration observed in *Arabidopsis* mutants (cpr1; constitutive expression of PR gene 1; Bowling et al., 1994, cpr5; constitutive expression of PR gene 5; Bowling et al., 1997 and agd2; aberrant growth and death; Rate and Greenberg, 2001) is responsible for various growth phenotypes. GA on the other hand plays a role in cellular differentiation and development. GA causes hyper-elongation of stems by stimulating both cell division and cell elongation, bolting in long day plants, induction of seed germination, fruit and flower development (Hedden and Philips, 2000). *GA$_2$ox* gene encodes gibberellin 2-oxidase, an enzyme which is responsible for the deactivation of active forms of gibberellins. Dijkstra et al. (2008) has shown that constitutive overexpression of *GA$_2$ox* from *Phaseolus coccineus* in *Solanum* species has resulted in a range of dwarfed phenotypes what corresponds to the findings presented here.

MATERIAL AND METHODS

Gene Constructs

Both of the investigated genes were cloned in pCAMBIA vectors to be driven by 35S constitutive promoter. *A. thaliana MKS1* sequence was introduced into pCAMBIA 1301 vector (Andreasson et al., 2005) and *N. tabacum* GA_2ox (AB125232.1) sequence into pCAMBIA1303 vector. The T-DNA of both vectors contains hygromycin resistance gene. Both vectors were introduced into *Agrobacterium tumefaciens* strain GV3101.

Plant Material and Transformation

Plants of *Petunia hybrida* 'Famous Lilac Dark Vein' were provided by Selecta Klemm GmbH & Co. KG (Stuttgart, Germany). Tissue culture conditions, plant transformation and regeneration of transgenic shoots were performed as described in Gargul et al. (2013).

Molecular Evaluation of the Transgenic Lines

Genomic DNA of all transgenic lines was isolated with a Seqlab Kit (Sequence Laboratories, Göttingen, Germany). Approximately 12 μg of DNA from control and all investigated transgenic lines were used for Southern blot hybridization. *Hind*III digested DNA fragments (Thermo Scientific/Fermentas, Vilnius, Lithuania) were separated on agarose-gels and transferred to a membrane as previously described in Sriskandarajah et al. (2007). The DIG-labelled probe was prepared according to the manufacturer's protocol and targeted for the *AtMKS1* transgene sequence (forward: 5'CCAAAGACAACTGCA AACCA3'; reverse: 5'TGCTCACCAAATCCAATCAA3') and $NtGA_2ox$ transgene sequence (forward: 5'CCAAAGACAACTGCAAACCA3'; reverse: 5'TGCTCACCAAA TCCAATCAA3'). Hybridization with specific probes, post-hybridization and an estimation of the visualized fragments procedures were performed as described in Sriskandarajah et al. (2007).

For the transgene expression analysis, total RNA was isolated from 30 mg of plant tissue using Invisorb Spin Plant RNA Mini Kit (Invitek & Co./STRATEC Molecular, Birkenfeld, Germany) according to the manufacturer's protocol. First strand cDNA synthesis and RT-PCR reactions were performed as described in Gargul et al. (2013). RT-PCR reaction for *AtMKS1* expression evaluation was performed using MKS1-570 primer pair (forward: 5'CCAAAGACAACTGCAAACCA3'; reverse: 5'TGCTCACCAAATCC AATCAA3'), and for $NtGA_2ox$ expression with GA_2ox-571 primer pair (forward: 5'CCAAAGACAACTGCAAACCA3'; reverse: 5'TGCTCACC AAATCCAATCAA3'). The expression of the reference gene was evaluated with the use of CYP (cyclophilin, Mallona et al., 2010) primer pair (forward: 5'AGGCTCATCATTCCACCGTGT3'; reverse: 5'TCATCTGCGAACTTAGCA CCG3').

Evaluation of Plant Growth and Development

Non-transgenic control and transgenic *MKS1* and GA_2ox plants of *Petunia* were acclimatized in the greenhouse conditions and multiplied using tip cuttings with the same number of internodes. The measurements of the total stem length and number of nodes were taken weekly as described in Gargul et al. (2015). The chlorophyll concentration analysis was performed as described in Gargul et al. (2013). Evaluation of generative growth, flower induction and development was observed in long day conditions (16 h of light). Number of open flowers was evaluated daily for 50 days period.

Statistical Methods

Upon analysis the variables height and height/nodes ratio were log-transformed. The data were analyzed by linear mixed models as described in Gargul et al. (2013). The data of the reproductive growth evaluation were analyzed by Wilcoxon test for the pairwise comparisons between clones with Holm-method modification. The statistical analysis was made with the R 2.12.1 program (R Development Core Team, 2010).

RESULTS AND DISCUSSION

Transgene Integration and Expression

Southern blot hybridization of 3 selected *MKS1*- (M1-M3) and *GA$_2$ox*-transgenic lines (G1-G3) was performed. In *MKS1*-transgenic lines the probe revealed the integration of one copy of the transgene in M1 and M2 and four copies in M3 clones. The difference in the number of integrated transgene between M1, M2 (1 copy) and M3 (4 copies) clones seemingly do not induce visible differences in phenotype between transgenic clones. All 3 investigated *GA$_2$ox*-transgenic clones contained one copy of the transgene (data not shown).

RT-PCR of all transgenic lines showed positive amplification of the targeted sequence of the transgene. RT-PCR in non-transgenic control plants did not produce amplification signal (data not shown).

Vegetative Growth Phenotype Analysis

The total stem length was approximately 2-fold decreased in *MKS1*-transgenic lines, while the stem lengths of *GA$_2$ox*-transgenic lines were approximately 6-fold decreased in comparison to the non-transgenic control plants (Fig. 1). The lengths of the internodes in *MKS1*-transgenic lines were 2.5-fold and in *GA$_2$ox*-transgenic lines 6.5-fold reduced in comparison to the non-transgenic plants, while the number of nodes in both *MKS1* and *GA$_2$ox*-lines was significantly increased (Tables 1 and 2). Andreasson et al. (2005) has reported that constitutive overexpression of *MKS1* gene in *Arabidopsis* resulted in approximately 4-fold increase of the salicylic acid (SA) concentration in the fresh weight of the leaf tissue while compared with control plants. Moreover, *Arabidopsis* plants exhibited semi-dwarfed phenotype similar to what we observed with *MKS1*-transgenic *Petunia* clones. It has been observed in several different studies, mainly in *Arabidopsis*, that plants with elevated SA concentration levels exhibit compact phenotypes (Petersen et al., 2000; Bowling et al., 1994), whereas plants overexpressing salicylate hydroxylase (*nahG*), exhibiting low levels of SA are more elongated (Gaffney et al., 1993). Constitutive overexpression of *GA$_2$ox* gene results in dwarfed phenotype in *Oryza sativa* (Sakai et al., 2003), *Nicotiana sylvestris* (Lee and Zeevaart, 2005) or *Arabidopsis thaliana* (Hedden and Phillips, 2000).

The morphology of the roots in both *MKS1* and *GA$_2$ox*-transgenic lines was not different from that of the non-transgenic control plants. The size and the shape of the leaves in both *MKS1* and *GA$_2$ox*-transgenic lines were not influenced. Nevertheless, in case of all three *GA$_2$ox*-transgenic lines there was a significant increase of the chlorophyll concentration (ranging from 2.16 to 2.43 mg g^{-1} per fresh weight) in comparison to the non-transgenic control plants (1.15 mg g^{-1} per fresh weight) (Table 2). It has been reported in several studies that plants with a lower level of physiologically active GA exhibit visibly darker leaves (Biemelt et al., 2004; Dijkstra et al., 2008). It might possibly be accredited to the reduced cell size but not reduced number of chloroplasts per cell. GAs influence the plant cell elongation (Richards et al., 2001; Keyes et al., 1990; Rood et al., 1990), through the increased expression of the xyloglucan endotransglucosylase gene, which encodes an enzyme responsible for the re-joining and cleavage of the cell wall hemicelluloses (xyloglucans) involved in cell wall expansion (Jan et al., 2004; Bourquin et al., 2002).

Delayed Flowering Phenotype

The flower morphology of both *MKS1* and *GA$_2$ox*- transgenic lines was not changed in comparison to those of non-transgenic lines. *MKS1*-transgenic lines of *Petunia* exhibited approximately 6 days and *GA$_2$ox*-trasgenic lines 7 to 12 days delay in opening of the first flower. However, the number of flowers at the 50th day of measurements was significantly increased in both cases. It has been reported that SA positively regulates flower induction as a part of the stress induced flowering phenomena in *Pharbitis nil* (Wada et al., 2010a) and *Perilla frutescens* var. *crispa* (Wada et al., 2010b). We speculate

that delayed flowering might occur due to the altered ethylene balance. Ethylene is involved in transition from vegetative growth to flowering (Ogawara et al., 2003; Abeles et al., 1992). Elevated SA levels have been reported to negatively influence the ethylene biosynthesis (Fan et al., 1996; Roustan et al., 1990). High levels of SA concentration might negatively influence the endogenous ethylene concentration, which might diminish the ethylene influence on flower induction.

It has been shown that increased levels of GA concentration in *Arabidopsis* plants that constitutively express *GA_{20}-ox* induces early flowering (Huang et al., 1993; Coles et al., 1999), whereas reduced concentration of active forms of GA in plants results in delayed flowering in *Silene armeria* (Cleland and Zeevaart, 1970) or *Arabidopsis* (Curtis et al., 2005) what corresponds to the presented results here.

CONCLUSIONS

Constitutive overexpression of *AtMKS1* and *$NtGA_2ox$* significantly reduced stem length and length of internodes during vegetative stage. Overexpression of *$NtGA_2ox$* resulted in visibly darker leaves due to 2-fold increase of chlorophyll concentration in the leaf blades of transgenic lines. The appearance of the first flower in both *AtMKS1* and *$NtGA_2ox$*-transgenic lines was significantly delayed, a feature that is unfavorable for commercial exploitation of the transgenic lines.

ACKNOWLEDGEMENTS

This project was supported by a Ph.D. research grant from the German Academic Exchange Service (DAAD) (Ref.: 323, PKZ: A/06/07504), which is gratefully acknowledged. Professor John Mundy at the University of Copenhagen, with approval from PBL, supplied the pCAMBIA1301 vector containing *AtMKS1* gene, which is also gratefully acknowledged.

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Tables

Table 1. Phenotypic evaluation of *Petunia MKS1*-transgenic lines.

	Control	M1	M2	M3
Total stem length of the plant (cm)	19c	10.2b	9a	9a
Length of the internodes (cm)	1.19c	0.51b	0.45a	0.45a
Number of nodes	15.95a	19.8b	20.32c	20.35c
Time to the anthesis of the first flower (days)	1a	6b	6.5b	6.5b
Number of flowers at 50th day of the observations	7.2b	6.57a	12.62c	12.65c

Letters (a, b, c) are significantly different at P<0.05 by Tukey's multiple range test.

Table 2. Phenotypic evaluation of *Petunia GA$_2$ox*-transgenic lines.

	Control	G1	G2	G3
Total stem length of the plant (cm)	23.46a	4.36b	3.78b	3.67b
Length of the internodes (cm)	0.81a	0.125b	0.12bc	0.105c
Number of nodes	29.42c	33.3a	31.45b	34.25a
Time to the anthesis of the first flower (days)	1a	7b	7b	12.5c
Number of flowers at 50th day of the observations	8.45a	10.97c	10.32c	9.4b
Chlorophyll per fresh weight (mg/g)	1.15a	2.16b	2.37b	2.43b

Letters (a, b, c) are significantly different at P<0.05 by Tukey's multiple range test.

Figures

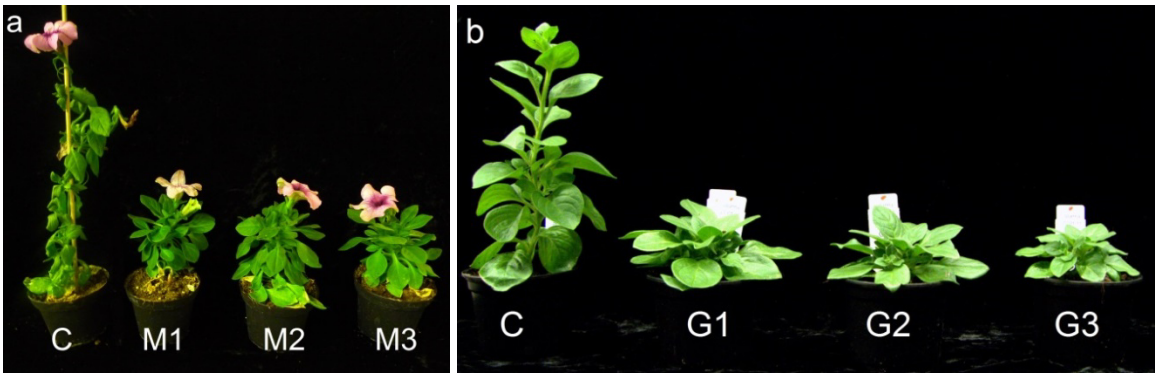

Fig. 1. Control and transgenic *Petunia* lines grown under greenhouse conditions. C – control, a) M1-M3 transgenic line with up-regulated *MKS1* gene, b) G1-G3 transgenic line with up-regulated *GA$_2$ox* gene.

New strategies for induction of compact growth in *Kalanchoë* flowering potted plants

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Abstract

The discussion and restrictions over the usage of the chemicals in European crop and ornamental plant production implies the search for novel substitute methods. In the present study, we investigate two alternative strategies to induce compact growth in *Kalanchoë*. First, we examined the possibility of using ethanol as an alternative plant growth regulator. In parallel, we follow a strategy of generating and investigating transgenic plants. All tested genotypes showed a correlation between the ethanol concentrations and compact growth after ethanol watering. However, high ethanol concentrations (more than 2%) led to leaf damage and delayed flower development in some genotypes. The use of ethanol as a growth regulator for ornamentals has several advantages. Ethanol is a biodegradable molecule that is inexpensive, easy and safe to apply, and non-toxic in the concentrations required. Significant growth retardation was also achieved in transgenic plants of *Kalanchoë blossfeldiana* with up-regulated *Arabidopsis thaliana MKS1* and *Nicotiana tabacum* GA_2ox genes. Both kinds of transgenic lines exhibited approximately 2-fold decrease in the stem length as well as in the length of the internodes. The number of nodes in both cases did not differ from the wild type control or was increased in comparison to the wild type.

Keywords: ethanol, MAP kinase substrate 1, gibberellin 2-oxidase, growth retardation, dwarfed phenotype

INTRODUCTION

Achieving compact growth of *Kalanchoë blossfeldiana* plants by alternative strategies is of a special interest of the *Kalanchoë* breeders and growers. *Kalanchoë* is one of the top selling plant in European potted plant market. *Kalanchoë* generated the turnover of EUR 55 million, with a number of plants sold per year of approximately 77 million in 2012 (Flora Holland, 2013). The consumers prefer pot plants with a compact growth habit. Such plants are traditionally produced by manipulation of the cultivation conditions (light and temperature), pruning, nutrition, irrigation and mainly application of chemical growth retardants. The hazard of using chemical growth retardants such as: Chlormequat ((2-chloroethyl) trimethylammonium chloride) or Daminozide has been under a debate in Europe recently. Many countries have already prohibited their usage due to the toxicity to humans and negative impact on the environment (Rademacher, 2000). *Kalanchoë* plants develop elongated internodes over time and also elongated inflorescence stem, therefore several different application regimes of chemical growth retardants are used for different *Kalanchoë* cultivars before the plants reach the consumers in order to maintain the favourable compact phenotype (Mibus et al., 2014; Gargul et al., 2013, 2015).

Elongation growth in plants is primarily controlled by gibberellic acid (GA) (Pimenta Lange and Lange, 2006). The genetic modifications to ornamentals and the use of synthetic growth regulators have mainly been aimed at the reduction of GA content by blocking GA synthesis-related enzymes. To reduce the GA concentration in transgenic *Kalanchoë*, an alcohol inducible promoter system was used to control the silencing of GA activating enzymes (GA_2ox) (Topp et al., 2008). Unexpectedly, the results demonstrated that the

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Acta Hortic. 1104. ISHS 2015. DOI 10.17660/ActaHortic.2015.1104.23
XXIX IHC – Proc. Int. Symp. on Ornamental Horticulture in the Global Greenhouse
Ed.: R.A. Criley

ethanol treatment of non-transgenic *Kalanchoë* led to a significant growth reduction. In another study Miller and Finan (2006) showed that in *Narcissus tazetta* 'Ziva' ethanol concentrations of 1 to 5% in the root-zone reduced plant height without causing a visible phytotoxicity to the roots. Accordingly, in the present experiments, we examined as one alternative strategy the possibility of using ethanol as a growth regulator in *Kalanchoë*.

As second alternative strategy we have focused our research on observing the phenotype of *Kalanchoë* with constitutive overexpression of genes reported to have growth-retarding influence on the phenotype in other species. Here we compare the effect of *MKS1* (MAP kinase substrate 1) and *GA_2ox* (gibberellin 2-oxidase) under 35S promoter in transgenic *Kalanchoë* lines. *MKS1* gene has been studied in *Arabidopsis thaliana* by Andreasson et al. (2005) who has shown that constitutive overexpression of *MKS1* leads to the compact phenotype, elevated levels of pathogenesis-related protein 1, increase of the salicylic acid (SA) levels and increased resistance to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*). *GA_2ox* however, is an enzyme which belongs to the group of dioxygenases, which are involved in the maintenance of the gibberellin homeostasis. They are responsible for the deactivation of GA1, GA4 active forms and their precursors into inactive molecules by 2-b-hydroxylation (Yamaguchi, 2008; Hedden and Phillips, 2000). Several studies show that GA-deficient mutants exhibit dwarfed phenotype in comparison to the wild type. It has been shown that the constitutive overexpression of *GA_2ox* results in dwarf phenotypes among others in *Arabidopsis thaliana* (Thomas et al., 1999; Hedden and Phillips, 2000; Wang and Li, 2005), *Oryza sativa* (Sakamoto et al., 2001, 2003) or a *Populus tremula* × *Populus alba* hybrid (Busov et al., 2003).

MATERIALS AND METHODS

Plant material

Plants of *Kalanchoë blossfeldiana* 'Molly', '1998-469', and 'African Pearl', as well as two species, *K. pubescens* and *K. campanulata*, were obtained from Knud Jepsen A/S (Hinnerup-Denmark), while *K. blossfeldiana* 'Sylt' was obtained from Dehne Topfpflanzen GmbH & Co. KG (Wismoor-Germany). Cuttings were rooted in a 10 cm-diameter pot with commercially produced soil 22/20°C (day/night) and day length was extended to 16 h by SON-T lamps (Osram, 400W, Philips Co.) that supplied 100 µmol m^{-2} s^{-1}. For flower induction and development, the plants were transferred to short day (SD) condition with 8 h light for additional 11 weeks. Leaf tissue of the cultivar '1998-469' was harvested, cultured and regenerated shoots were maintained under tissue culture conditions as described by Ilczuk et al. (2009).

Ethanol treatment by watering

Watering solutions contained 0, 0.5, 1, 2 or 4% (v/v) ethyl alcohol (type 520) denatured with 1% petroleum (Fa. Sonnenberg GmbH & Co KG, Braunschweig, Germany). Two-week-old rooted cuttings received a 50 mL ethanol solution or water weekly for 16 weeks. The excess solution draining from the pot was collected in a saucer allowing to be soaked up by plants for 1 h.

Plant transformation and molecular characterization of the transgenic lines

MKS1 (*Arabidopsis*) and *GA_2ox* (*N. tabacum*, AB125232.1) sequence was cloned in front of a 35S promoter within a pCAMBIA1301 (Andreasson et al., 2005) respectively a pCAMBIA1303 vector. The T-DNA of both vectors contains a hygromycin resistance gene. Plant transformation, regeneration, selection and acclimatization of the transgenic lines was performed as described in Gargul et al. (2013). Genomic DNA of all transgenic *MKS1*- and *GA_2ox*-lines was isolated with a Seqlab Kit (Sequence Laboratories, Goettingen, Germany). 10 to 12 µg of DNA from control and all transgenic lines were used for Southern blot hybridization. *BamH*I digested DNA fragments (Thermo Scientific/Fermentas, Vilnius, Lithuania) were separated on agarose-gels and transferred to a membrane as described in Sriskandarajah et al. (2007). Hybridization with *MKS1* or *GA2ox* specific probes, post-

hybridization as described in Sriskandarajah et al. (2007).

Evaluation of plant growth and flower development

The experiments for ethanol treatment were conducted in a completely randomised design using five plants per treatment of each genotype with three replications. Plant height was measured in a weekly interval from the soil surface to the apical meristem. Flowering time was defined as the time from short day treatment start until the first open flower appeared. During the treatment plants were cultivated and evaluated for 16 weeks (4 weeks LD +12 weeks SD). Additional first flower data were collected during the entire period of SD condition. Lines of *MKS1* and *GA_2ox*-transgenic plants were multiplied by cuttings with the same number of internodes. The measurements of the total height and number of internodes were made weekly.

Statistical methods

The data were analyzed by linear mixed models. The time, replication and transgenic line were fixed factors. Before analysis the variables height and height/nodes ratio were log-transformed. After fitting the model, multiple comparison procedures (Hothorn et al., 2008) were applied to compare the mean of the transgenic lines to the mean of the control plants for each variable and pooled over the 2 replications. The statistical analysis was made with the R 2.12.1 program (R Development Core Team, 2010).

RESULTS AND DISCUSSION

Ethanol treatment

Weekly watering with 1 or 2% ethanol solution did not lead to any damages on leaves or roots in all investigated cultivars and species. *Kalanchoë pubescens* and *K. campanulata* were sensitive to the highest concentrations of 4% as shown by smaller and deformed leaves and weak stems (data not shown). All investigated cultivars of *Kalanchoë blossfeldiana* were able to tolerate the weekly 4% ethanol treatment without symptoms on leaves, roots or stems (Figure 1). In all tested genotypes no significant differences of node number was detectable after 4 weeks of treatments. After 16 weeks only 4% ethanol treatment lead to significant reduction of node number in 'Molly', *K. pubescens* and *K. campanulata*. In all other *K. blossfeldina* cultivars no significant differences of node number were detectable after 16 weeks (data not shown). All tested genotypes showed significant correlation between ethanol concentration and length of the internodes in the end of the experiment after 16 weeks (Figure 1). In agreement with Topp et al. (2008), the present study showed similar reductions in internode lengths in *Kalanchoë.* Several hypotheses may explain the mechanism by which root-zone ethanol influences plant growth. The first hypothesis is based on a simple osmotic effect of the ethanol in the root-zone, which causes reduced water uptake and reduced turgor. This hypothesis was put forward to explain the action of root-zone ethanol as a growth retardant in *Narcissus tazetta* (Miller and Finan, 2006) and could explain the physiological action of ethanol concentrations higher than 2%. The second hypothesis is based on the direct ethanol toxicity in the cells of the root surface. Investigations with *Narcissus* demonstrated that ethanol concentrations between 1 and 5% could effectively reduce height without visible damage to the roots or other plant organs (Miller and Finan, 2006). Unfortunately, no investigation was performed at the cellular level in these experiments to verify the hypothesis of direct ethanol toxicity on the root cells. In other studies on tobacco (Caddick et al., 1998), the roots were drenched with 0.01-2% ethanol solutions for two weeks without any reported physiological consequences.

After a six-week LD period investigated plants were cultivated under SD for flower induction. Ethanol watering of the cultivars 'Molly' and '1998-469' with concentrations lower than 2% had no influence to the time of opening the first flower. However, the treatment of 4% ethanol leads to longer time to open the first flower in cultivars 'Molly' and '1998-469' (Figure 2). Both cultivars 'African Pearl' and 'Sylt' needed more time to open the first flower after the treatment with more than 2% ethanol. Already the lowest

concentration of 1% ethanol extends the time of opening the first flower in *K. pubescens* (Figure 2). This kind of delay of flower induction after ethanol treatment was never described and investigated in other plants. Also in other *Kalanchoë blossfeldiana* cultivars non-significant delay in flower induction after 2% ethanol watering was detected (Topp et al., 2008).

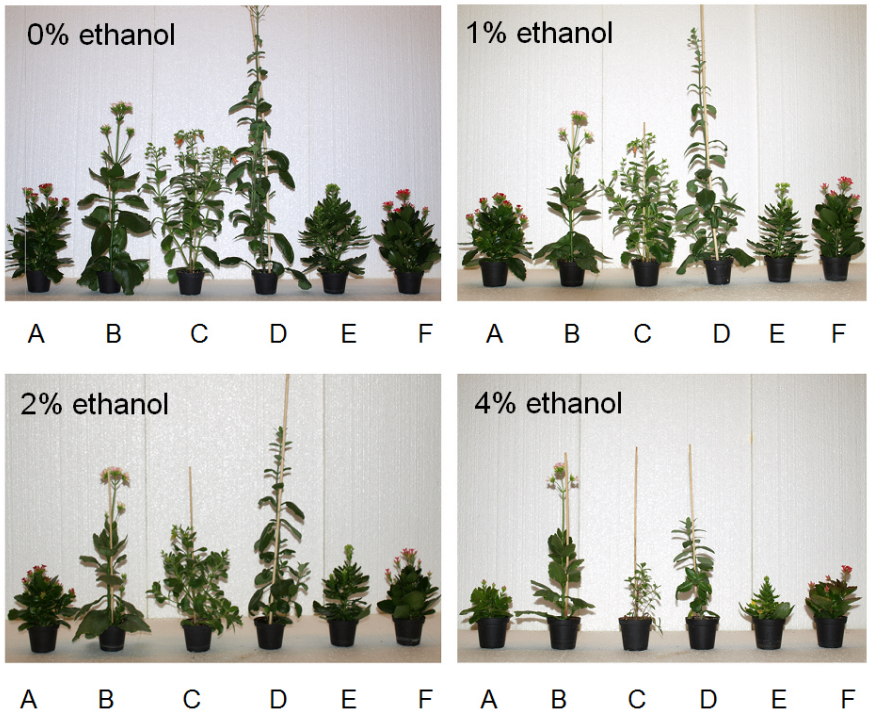

Figure 1. Demonstration of representative ethanol (0, 0.5, 1, 2, and 4%) treatment of *Kalanchoë blossfeldiana* cultivars (A) 'Molly', (B)'1998-469', (C) *Kalanchoë pubescens*, (D) *K. campanulata*, (E) 'African Pearl' and (F) 'Sylt' and after 4 weeks LD and 12 weeks under SD condition.

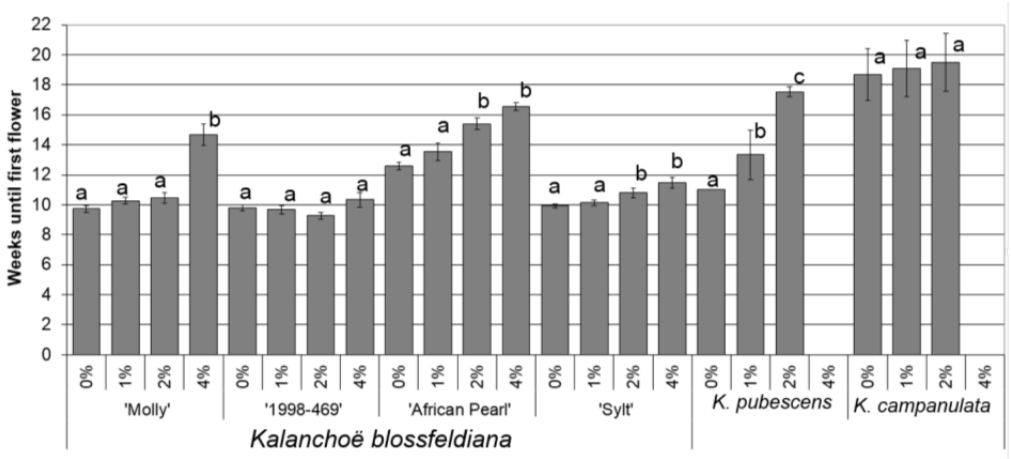

Figure 2. Influence of different ethanol concentrations (0, 1, 2 and 4%) on first open flower of *Kalanchoë blossfeldiana* 'Molly', '1998-469', 'African Pearl' 'Sylt', *Kalanchoë pubescens* and *K. campanulata* under SD condition (*n*=15).

In conclusion, ethanol has several advantages as a growth regulator for ornamental plants. It is a biodegradable molecule that is inexpensive, easy and safe to apply, and non-toxic at the required concentrations.

Genetic transformation

Southern blot hybridisation showed one copy of the transgene integrated in the M1 and M2 (M – lines with *MKS1*) transgenic lines and 2 copies in the M3 line. In case of plants with overexpressed *GA₂ox*, one copy of the transgene integrated in the genome of G1 transgenic line, 7 copies in the genome of G2 line, and 2 copies in the genome of the G3 line. In all transgenic lines the expression of the transgene was confirmed by the RT-PCR. Constitutive overexpression of both genes, *MKS1* and *GA₂ox* in *Kalanchoë* resulted in reduced growth phenotype (Figure 3). *Kalanchoë* transformed with *MKS1*-construct exhibited more than 2-fold decreased stem and internode lengths in comparison to the control non-transgenic plants, while the number of nodes did not differ significantly in most investigated clones (Figure 3a). Results of the studies in *Arabidopsis* show that plants with increased SA levels exhibit compact phenotypes. Bowling et al. (1994, 1997) reported that cpr1 (constitutive expression of PR gene 1) and cpr5 (constitutive expression of PR gene 5) mutants which have constitutively high SA level exhibit dwarf-like phenotypes, which is caused by the SA negative affect on the cell size and endo-reduplication ability. The contrary effect was observed in plants with reduced SA level in plants which overexpress *nahG* gene, which encodes salicylate hydroxylase. This enzyme converts SA to catechol. Those plants with low levels of SA and exhibit higher growth rate (Abreu and Munné-Bosch, 2009; Du et al., 2009).

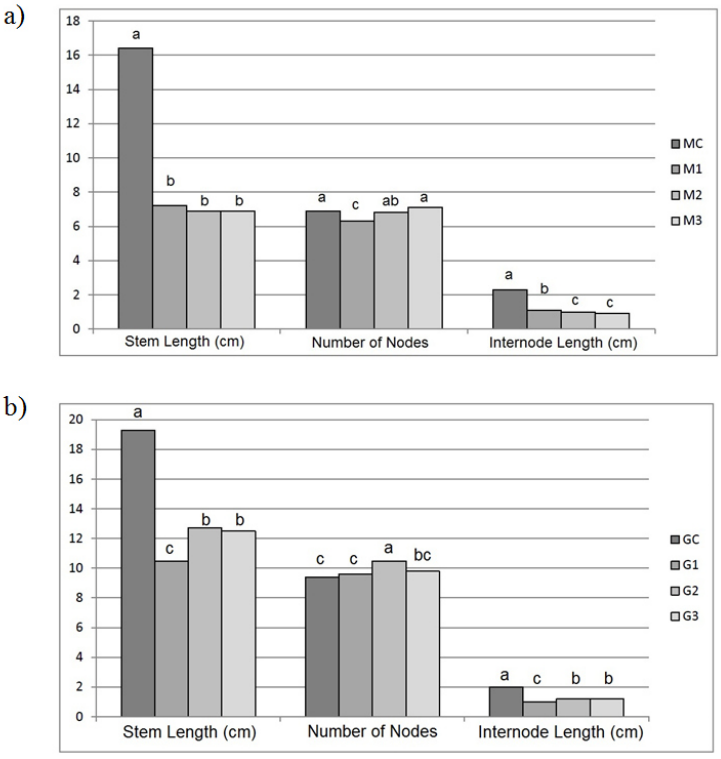

Figure 3. Traits of *Kalanchoë blossfeldiana* '1998-469'control plants and a) 35s::MAP kinase Substrate 1 (*MKS1*) and b) 35s::gibberellin 2-oxidase (*GA₂ox*) transgenic lines: Mean plant length (cm); mean number of nodes per plant and mean internode length (cm). Bars marked with letters (a, b, c) are significantly different at $P<0.05$ by Tukey's multiple range test. Bars represent the mean ± SD (n=20).

Kalanchoë lines with overexpressed *GA_2ox* gene exhibited less than 2-fold decreased stem and internode lengths in comparison to the wild type plants, while the number of nodes was comparable or higher than in the wild type plants (Figure 3b). Dijkstra et al. (2008) showed similar results in *Solanum melanocerasum* and *Solanum nigrum* transgenic plants with up-regulated *$PcGA_2ox1$*. Some clones of the transgenic lines of *S. melanocerasum* showed even 16-fold decrease in stem length in comparison to the wild type plants, while *S. nigrum* lowest values exhibited approximately 5.5-fold decrease in stem length. Another example of the up-regulation of *GA_2ox* effect on the growth rate is overexpression of *$OsGA_2ox1$* gene in *Oryza sativa*, which resulted in approximately 6-fold decrease in stem length in transgenic rice plants in comparison to the wild type plants (Sakamoto et al., 2001).

Constitutive overexpression of both *MKS1* and *GA_2ox* gene results in dwarfed-like phenotype in *Kalanchoë blossfeldiana*, therefore using molecular-transgenic methods in creating compact potted plants may be considered in the future.

ACKNOWLEDGEMENTS

Part of this project was supported by a Ph.D. research grant from the German Academic Exchange Service (DAAD) (Ref.: 323, PKZ: A/06/07504), which is gratefully acknowledged. The authors would like to thank Prof. John Mundy (University of Copenhagen, Denmark) for kindly providing the binary vector containing the *AtMKS1* sequence, with approval from Plant Bioscience Ltd. (PBL), with which the part of results were obtained.

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Poster

Plant growth regulation by constitutive overexpression and silencing of *GA$_2$ox* in *Petunia hybrida*.

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Introduction

Establishing an alternative way of creating compact plants instead of the chemical growth retardants application is one of the top targets of recent breeding programs in ornamental plants. Here we describe work on *Petunia hybrida*, which is considered to be a model plant among ornamental species. The manipulation of the gibberellin metabolism of the plants was chosen as a strategy for the modification of the growth habit.

Results

Plants of *Petunia hybrida* 'Famous Lilac Dark Vein' were transformed with the pCAMBIA1303 modified vector, containing a construct with the *gibberellin 2-oxidase* (*GA$_2$ox*) gene from *Nicotiana tabacum* under the control of the Ca35S promoter. The transformation was performed with the use of *Agrobacterium tumefaciens* strain GV3101. The expression and integration of the transgene was analysed using reverse-transcription (RT)-PCR and Southern blot analysis (Fig. 1). The mean lengths of the transgenic lines were four-fold shorter than those of the wild-type plants, while the mean numbers of nodes were either similar or higher in the transgenic lines than in the wild-type plants (Fig. 2 and 3). The flowering delay was observed with a range of 7 to 12 days from the anthesis of the first open flower in wild type. The flower morphology of the transgenic lines did not differ from wild-type plants. Moreover transgenic lines exhibited darker green pigmented leaves containing an approximately two-fold increase in chlorophyll content over the wild-type control plants (Fig. 3).

Plants of *Petunia hybrida* 'Fantasy Blue' were submitted to Virus Induced Gene Silencing (VIGS) experiments with the *Nicotiana GA$_2$ox* gene cloned into tobacco rattle virus vectors (TRV). Plants with down regulated *GA$_2$ox* gene exhibited a phenotype with elongated stem. Expression analysis with the use of real time RT-PCR method showed significantly lower expression level of the *GA$_2$ox* gene after 4 weeks post inoculation. (Fig. 3f and 4)

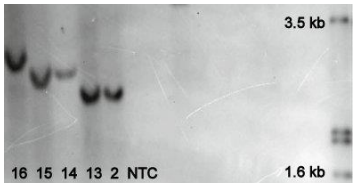

Fig. 1 Southern blot autoradiogram of transgenic lines of *P. hybrida*. The genomic DNA was digested with *HindIII* and probed with a DIG-labelled probe that was homologue to the part of *HptII* gene, which was a part of the transfered T-DNA. NTC—non-transgenic control.

Fig. 2 Representative *P. hybrida* 'Famous Lilac Dark Vein' flowering control and transgenic lines grown under greenhouse conditions.

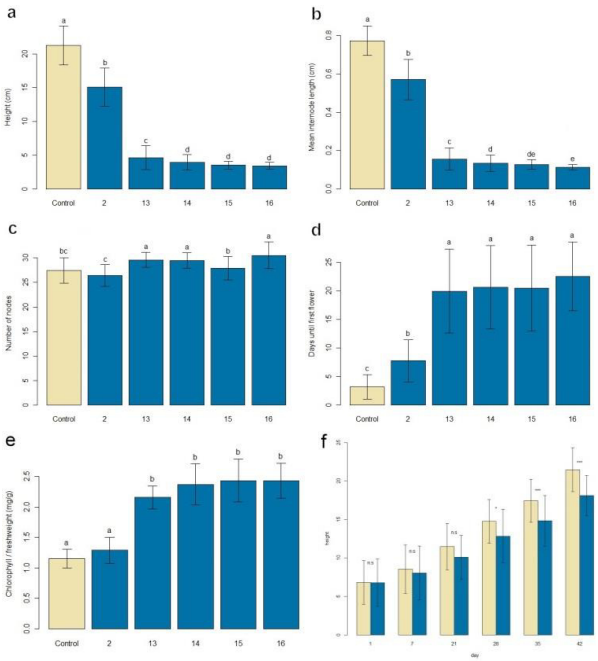

Fig. 3 Vegetative and generative traits of the *P. hybrida* control plants and 35S::*gibberellin 2-oxidase* (*GA$_2$ox*) transgenic lines (a-e): a) Mean plant length (cm); b) mean internode length (cm); c) mean number of nodes per plant; d) mean number of days until first open flower (anthesis); e) chlorophyll concentration (mg/g of fresh weight). Bars marked with letters (a, b, c, d, e) are significantly different at P<0.05 by Tukey's multiple range test. f) relative concentration of *GA$_2$ox* transcript in TRV1-GUS – control (grey) and TRV1-*GA$_2$ox* (blue) treated plants over time. Signif. codes: n.s. p. value > 0.05; * 0.05 > p.value > 0.01; ** 0.01 > p.value > 0.001; *** 0.001 > p.value > 0.0001 . Bars represent the mean ± SD (n = 20) in all the experiments.

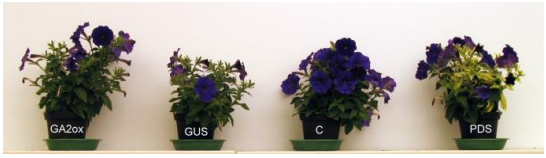

Fig. 4 Representative *P. hybrida* 'Fantasy Blue' after 4 weeks post inoculation. GA2ox – plant inoculated with TRV1-*GA$_2$ox*, GUS – plant inoculated with TRV1-GUS gene, as a control of the phenotype; C – non treated plant; PDS – plants inoculated with TRV1-PDS gene, as a control of the TRV1 vector action.

Gargul, JM., Mibus H. and Serek M. 2013 Constitutive overexpression of *Nicotiana GA2ox* leads to compact phenotype and delayed flowering in *Kalanchoë blossfeldiana* and *Petunia hybrida*. Plant Cell, Tissue and Organ Culture 115: 407-418.

Discussion

Discussion

The objective of this study was to create compact *Kalanchoë* and *Petunia* plants with the use of *Agrobacterium*-mediate gene transformation method. Phenotypic effects of *GA$_2$ox* and *MKS1* gene overexpression were evaluated in transgenic plants in both species. Also virus-induced gene silencing method of *GA$_2$ox* and *MKS1* genes was applied in *Petunia* and possible modification of the stem growth was observed. Additional observations for pathogen-tolerance were made for *MKS1*-transgenic *Petunia* plants.

Evaluation of the vegetative growth in 35S::*GA$_2$ox*-, *dBI::GA$_2$ox*- and 35S::*MKS1*-transgenic plants

All of the presented here strategies for growth retardation resulted in significant growth reduction in both studied species, *Kalanchoë* and *Petunia*. The vegetative growth retardation in both cases of *GA$_2$ox*- and *MKS1*-mutants is a result of the change in phytohormonal balance. In *GA$_2$ox*-transgenic plants it is the reduced level of active gibberellins. And in transgenic *MKS1* plants the reason for reduced stem growth is elevated level of salicylic acid.

Constitutive overexpression of *GA$_2$ox* resulted in average 1.8-fold decrease of the total stem length and 2-fold decrease in length of the internodes in *Kalanchoë* transgenic clones in comparison to non-transgenic control plants. Similar results were observed in *Kalanchoë* transformed with *GA$_2$ox* driven by *dBI* promoter. The average length of the stem was decreased 2.3-fold and the length of the internodes was decreased 2.5-fold on average. The number of nodes was similar or higher in clones transformed with 35S::*GA$_2$ox* and *dBI*::*GA$_2$ox* constructs in comparison to non-transgenic control plants. 35S::*GA$_2$ox*-transgenic *Petunia* plants exhibited 6-fold decrease of the stem length, whereas *dBI::GA$_2$ox*-transgenic *Petunia* plants resulted in approx. 3-fold reduction of stem length in comparison to the non-transgenic control plants. Only clone P2 35S::*GA$_2$ox*-mutant did not exhibit such extreme dwarfism effect of constitutive *GA$_2$ox* expression. That result might be caused by the specific features of the chromosomal environments, i.e.: the neighborhood of the silencers, where the T-DNA was integrated (Frizzi and Huang, 2010). Another explanation may be DNA-based metylation, somaclonal variation or spontaneous mutations. The southern blot revealed single-copy of the transgene integration in the P2 clones, what might also lead to the

predisposition of the transgene silencing what has been reported by Elmayan and Vaucheret, (1996). Except 35S::*GA$_2$ox*-transgenic *Petunia* line (P2), which did not show difference in phonotype in comparison to the non-transgenic plants. The magnitude of dwarfism effect was larger on *Petunia* plants than on *Kalanchoë* in both 35S:: and *dBI::GA$_2$ox*-transgenic plants. Also it might be concluded that 35S promoter leads to greater reduction in stem length in *Petunia*, but in *Kalanchoë* the reduction in stem length is similar in respect of the used 35S or *dBI* promoter.

Constitutive overexpression of *GA$_2$ox* was performed in *Solanum melanocerasum* by Djikstra et al. (2008) who reported a range of different phenotypes in different clones. While the length of the control plants stems were approx. 23 cm long, the stem length of transgenic plants with constitutive overexpression of *GA$_2$ox* ranged from 4.7 to 18.3 cm. Similar results were observed in *Solanum nigrum*, where the length of the stem ranged from 11.2 cm to 42.5 cm and the length of the stem in control non-transgenic plants was 62.6 cm. Ectopic expression of *Nerium oleander GA$_2$ox* in transgenic *Nicotiana* resulted in reduced level of active GA_1 and a range of dwarf phenotypes which were correlated with levels of the transgene expression (Ubeda-Tomas et al., 2006). The height and length of the internodes of adult transgenic plants was significantly reduced with different degree of reduction. The average internode length in transgenic lines values of 0.4 +/- 0.1 mm and in control plants of 25.6 +/- 3.5 mm. The number of the internodes on 6-month-old transgenic plants was higher or similar to that of control plants. In addition one out of three investigated transgenic *Nicotiana* lines was delayed in time of germination. Also one line germinated with lower rate than that of control. Similarly *Nicotiana sylvestris* with ectopic overexpression of *GA$_2$ox$_3$* from *Spinacia oleracea* exhibited a range of dwarfed phenotypes and the severity of the phenotypes was correlated with the accumulation of the *SoGA$_2$ox$_3$* transcripts. The total stem length of the investigated transgenic plants was less than half of that of control non-transgenic plants and the number of nodes was almost doubled (Lee and Zeevaart, 2005). Different approach but based on the manipulation with GA signaling in *Petunia* was investigated by Liang et al. (2014). It was observed that silencing of *GID1*-like receptors genes has a strong impact on the plant architecture. Inoculated plants exhibited short branches and internodes. *Petunia* plants were also transformed with *GA-insensitive* mutant gene (*gai*) from *Arabidopsis* using glucocorticoid GVG-inducible system with dexamethasone (dex) as an inducer. GVG::*gai* plants were sprayed daily for 10 days with dex. The resulting overexpression of *gai* caused significant retardation of the plant growth and smaller leaf size

in comparison to the non-transgenic control plants (Liang et al., 2014). Although in this study the control non-transgenic plants treated with dex did not show abnormal phenotype, it has been reported in several different studies, that the application of dexamethasone on plants or amount of the active GVG in the nucleus is related to the severe growth defects and induces defense-related genes (Kang et al., 1999; Ouwerkerk et al., 2001; Andersen et al., 2003).

Constitutive overexpression of *Arabidopsis MKS1* gene resulted in approx. 2.3-fold decrease in stem length in transgenic *Kalanchoë* lines and 1.9-fold decrease in transgenic *Petunia* lines in comparison to the non-transgenic control plants. The number of nodes was similar in transgenic and control plants, and the length of the internodes was approx. two times shorter in transgenic lines of both species. Constitutive overexpression of the *MKS1* gene in *Arabidopsis* resulted in approx. 4-fold increase of SA concentration. It is possible that the reduced growth in *Kalanchoë* and *Petunia MKS1*-transgenic clones is a result of the high concentration of the SA. It has been reported in several species that elevated SA concentration leads to not only higher resistance to pathogens but also dwarfed phenotype. It was observed that in plants which accumulate higher levels of SA, the expression levels of *xyloglucan endotransglocosylase/hydrolase* (*XTH*) genes were distinctly down-regulated (Miura et al., 2010). Those genes encode enzymes involved in cell wall loosening, expansion and endo-reduplication ability (Rose et al., 2002). It can be concluded that higher SA levels might cause smaller cell sizes what results in a dwarf-like phenotypes. Such phenotypes were observed in *cpr1 - constitutive expression of PR gene 1*, *cpr5 - constitutive expression of PR gene 5*, *acd6-1 - accelerated cell death 6-1* and *agd2 - aberrant growth and death 2 Arabidopsis* mutants (Bowling et al., 1994, 1997; Rate et al., 1999; Rate and Greenberg, 2001) (Tab. 1).

Several cultivars of *Kalanchoë blossfeldiana* (*Kb*) produces undesirable elongated stem with loosely arranged leaves and also elongated inflorescence stem. These phenotypic traits strongly decrease the ornamental value of *Kalanchoë* as an ornamental potted indoor plant. There are several examples of molecular breeding strategies reported for different *Kb* cultivars towards compact growth. Christiansen et al. (2008) has shown that transformation with *Agrobacterium rhizogenes* of *Kb* cv. 'Molly' (Ri-lines) resulted in plant height reduction between 44.4 % and 51.9 % in comparison to the control plants, while the number of nodes was significantly higher and ranged from 9.1 cm to 14.4 cm in comparison to the control plants with average number of 8.3 nodes. The size of the leaves in Ri-lines was significantly reduced and wrinkled in comparison to the control plants. Another dwarfing strategy in *Kb*

cv. 'Molly' was based on genetic manipulation of GA biosynthesis by an ethanol induced promoter system controlling silencing of *GA_{20}oxidases* (*GA20ox*), which encode for enzymes which lead to GA synthesis (Topp et al., 2008). It has been observed that watering with 2 % ethanol itself resulted in dwarfed phenotype. In transgenic plants grown under long day conditions the reduction in the growth ranged between 63 % to 90 % in comparison to the control plants with normal watering. Whereas watering non-transgenic control plants with 2 % ethanol resulted in 66 % reduction of the growth in comparison to the non-transgenic plants with normal watering. This unexpected dwarfing effect of ethanol was later on investigated by Mibus et al. (2014). In this research the influence of ethanol was observed in several *Kb* cultivars where 'Molly' naturally exhibits fairly compact growth habit and '1998-469' cultivar naturally exhibits most elongated growth habit. Different ethanol concentrations were checked. After 16 weeks of weekly watering of the plants with 50 mL water solution of 4 % ethanol *Kb* cv. 'Molly' exhibited significant reduction in number of nodes in comparison to plants watered normally. For *Kb* cv. '1998-469' there were no significant differences in the number of nodes at that time. Another dwarfing possibility for different *Kb* cultivars was presented by Lütken et al. (2010). Eight different cultivars of *Kalanchoë* were transformed by *Agrobacterium tumefaciens* with a vector containing *Arabidopsis SHI* gene driven by 35S constitutive promoter. Most transgenic lines exhibited significant reduction in height. Most dwarfed phenotype was observed in transgenic clones of line '57' and 'Naomi' and 'Sarah' interspecific hybrids. Similar strategy where two KNOX genes (*KxhKN4, KxhKN5*) isolated from *Kalanchoë x houghtonii* were constitutively expressed in *Kalanchoë* cv. 'Molly'. Most transgenic lines exhibited dwarfed phenotype and deformed, dark-green leaves. Silencing of *KxhKN5* resulted in dwarfed phenotype as well (Lütken et al., 2011).

Phenotypic evaluation of the reproductive growth in 35S::*GA_2ox*- and 35S::*MKS1*-transgenic lines

Unfortunately the delay in flowering was observed in both species for constitutively expressing *GA_2ox* and *MKS1*-transgenic lines. The effect of GA on flowering is complex. It can be neutral, promotive or inhibitory (Zeevaart, 1976; Pharis and King, 1985). Several species of LD plants grown under non-inductive conditions by GA application can be induced to bolt and produce flowers (i.e.: biennial rosette species, Lang, 1956; Wittwer et al.,

1957). GA can act as an mobile inductive signal which interferes with the vegetative to generative state transition (King, 2012), therefore reduced levels of active GA leads to delay in flowering. 35S::*GA$_2$ox*-transgenic *Kalanchoë* lines exhibited significant delay in flowering up to 27 days on average, and transgenic *Petunia* up to 21 days on average except the P2 line. The length of the inflorescence stem of the transgenic *Kalanchoë* was 3-fold reduced in comparison to the inflorescence stem length of the control plants. The number of flowers at day 50 of the observations in transgenic *Kalanchoë* lines was 3-fold lower in comparison to control, but was still increasing. In transgenic *Petunia* at day 50 number of flowers was significantly higher than number of flowers in control plants and was increasing in transgenic plants while the maximum number of flowers in control plants was reached at approx. day 30 of the observations. 35S::*MKS1*-transgenic *Kalanchoë* plants exhibited 15-20 days delay in flowering and *Petunia* 6-11 days in comparison to the non-transgenic control plants. The length of the inflorescence stem of 35S::*MKS1*-transgenic *Kalanchoë* was 5-fold reduced in comparison to control plants and the number of flowers at day 50 was aprox. 2-fold lower than in control. In *MKS1*-transgenic *Petunia* plants the number of flowers at day 50 of the observations was either similar to the number of control plants for clones with one copy of the transgene integrated in the genome or approx. 2-fold higher in clones with 4 copies of the transgene in the genome than in non-transgenic control plants.

Constitutive overexpression of *MKS1* gene leads to the increase of SA levels (Andreasson et al., 2005). SA is considered to be a flower-inducing factor. Such regulation usually occur under abiotic stress conditions and it might be an aspect of species preservation mechanism (Wada et al., 2010 a, b). However in 35S::*MKS1*-transgenic *Kalanchoë* and *Petunia* plants a significant delay in flowering was observed. A possible explanation for this phenomena might be the influence of elevated SA levels on ethylene biosynthesis inhibiting the conversion of 1-aminocyclopropane-1-carboxylic acid (ACC) to ethylene (Leslie and Romani, 1986, 1988; Romani et al., 1989; Huang et al., 1993; Fan et al., 1996; Srivastava and Dwivedi, 2000). Ethylene advances the vegetative to reproductive state transition, and it is presupposed that the elevated SA levels cause low ethylene levels, which lessen its influence on flower induction. Also petals of 35S::*MKS1*-transgenic *Kalanchoë* lines had significantly higher anthocyanin concentrations in comparison to the non-transgenic control plants, which might be also explained by the elevated SA concentration. Similar effects were observed in grape vine, carrot or rose (Saw et al., 2010; Sudha and Ravishankar, 2003; Ram et al., 2013). The petals of transgenic *Petunia* lines did not differ in color from the non-transgenic plants.

Christiansen et al. (2008) observed in *Kb* cv. 'Molly' that the flowering was delayed in time in most Ri-lines and the delay ranged from 3 to 27 days in comparison to the control plants. The flower diameter was significantly decreased in Ri-lines. The total number of flowers per plant and the number of inflorescences was significantly reduced when compared to the control plants. In transgenic *Kb* cv. 'Molly' watered with 2 % ethanol described by Topp et al. (2008) the delay in flowering ranged between 2 to 16 days in comparison to the non-transgenic control plants with normal watering. The non-transgenic plants watered with 2 % ethanol exhibited 3-day delay in flowering in comparison to control plants with normal watering. In a similar study by Mibus et al. (2014) it has been shown that watering *Kb* cv. 'Molly' and '1998-469' cultivars with the concentration lower than 2 % ethanol did not influence the time of anthesis, but 4 % ethanol significantly delayed flowering in both cultivars. The average inflorescence length was reduced in 'Molly' when watered with 0.5 % or higher concentration of ethanol, whereas in '1998-469' cultivar a significant reduction in inflorescence stem was observed while watered with 1 % or higher concentrations of ethanol. Lütken et al. (2010) has shown that constitutive overexpression of *SHI* gene from *Arabidopsis* in different *Kalanchoë* cultivars like 'Molly' did not influence flowering time, except of several transgenic clones of '57' line and interspecific hybrids of 'Naomi', 'Sarah' and 'Suzanne'. However it was observed in one of the investigated transgenic lines that expression of *AtSHI* gene driven by its own constitutive promoter in *Kb* cv. 'Molly' leads to approx. 4 days earlier flowering, but the vegetative growth did not differ from *Kb* cv. 'Molly' *SHI*-lines controlled by 35S promoter. Overexpression of *KNOX* genes in *Kb* cv. 'Molly' resulted in delay of 1-2 weeks in flowering in several transgenic lines or did not influence the flowering time in others in comparison to the non-transgenic control (Lütken et al., 2011). *Petunia* plants with modified GA signaling by silenced *GID1*-like genes exhibited also significantly delayed flowering (Liang et al., 2014).

Leaf morphology in plants with constitutive and stem-specific *GA_2ox* expression

Chlorophyll concentration was measured in *Kalanchoë* and *Petunia* plants with constitutively overexpressed *GA_2ox*. In both species the chlorophyll concentration levels were approx. 2-fold elevated except of P2 line. Nevertheless, except the visual darker color of the leaves in

35S::*GA$_2$ox* transgenic plants, the leaves did not exhibit other morphological differences in comparison to the control plants. In contrast, to those observations, clones which overexpress *GA$_2$ox* driven by *dBI* promoter, exhibited similar color to this of the WT, but the morphology was changed. Leaves of clones of both species were approx. 2-fold smaller and the petiole was reduced. Leaves of *Kalanchoë* were also convex. This effect might be explained by the *GA$_2$ox* expression in the main vascular bundles of the leaf and petiole, which is possibly similar to the expression of the GUS reporter gene in the corresponding construct. Leaf color and morphology are next to the flower shape, color or scent and general plant growth habit one of the qualities which are important in ornamental plants. It has been reported for several plant species that decreased active GA concentration levels correspond to the increased chlorophyll concentration (Biemelt et al., 2004; Ubeda-Tomas et al., 2006; Dijkstra et al., 2008). This effect possibly is the consequence of the smaller cell size in transgenic plants which contain the same number of chloroplast per cell. The influence of GA on promoting cell growth and division has been reported in several studies (Richards et al., 2001; Jupe et al., 1988; Keyes et al., 1990; de Souza and MacAdam, 2001; Rood et al., 1990). It might be explained by the correlation between the decreased GA levels and increased expression of the *xyloglucan endotransglucosylase* gene (*XET*) (Uozu et al., 2000). Xyloglucan endotransglucosylase is an enzyme responsible for the cleavage and re-joining of the primary cell wall hemicelluloses, which play a crucial role in regulating cell wall expansion (Jan et al. 2004; Bourquin et al. 2002). Similar phenotypic characteristics, dark-green and deformed leaves were observed in *Kalanchoë* 'Molly' which constitutively overexpress *KNOX* genes (Lütken et al., 2011) and in *Petunia* plants with silenced *PhGID1A, PhGID1B* or *PhGID1C* genes (*GA-insensitive dwarf1*)(Liang et al., 2014).

Petunia phenotype in plants with downregulated *GA$_2$ox* and *MKS1* genes

The downregulation of both genes, *GA$_2$ox* and *MKS1* have been performed with the use of virus-induced gene silencing method. As expected silencing of the *GA$_2$ox* caused significant elongation of the stem lengths in the inoculated *Petunia* plants in comparison to control. Low levels of *GA$_2$ox* transcript indicated low level of *GA$_2$ox* expression what possibly influenced low activity of GA-2-oxidase enzyme. Therefore it might be assumed that the observed increase in stem length is a result of higher levels of active forms of gibberellins in plants

with silenced *GA_2ox* gene. Silencing of *GA_2ox* in *Nicotiana tabacum* with the use of hairpin RNAi silencing vector lead to the approx. 1.6-fold increase in stem length (Dayan et al., 2010). Stem growth elongation might be beneficial for commercial uses as ground cover plants or hanging baskets for several *Petunia* cultivars i.e.: 'Fantasy Blue' or 'Picobella Blue' which exhibit compact growth habit.

Similar approach in stem growth modification based on gene silencing was undertaken in *Petunia* where silencing of *GDI*-like genes lead to growth retardation (Liang et al., 2014) and overexpression of antisense fragment of GA activating enzymes in *Kalanchoë* also resulted in dwarfed-phenotypes of transgenic lines (Topp et al., 2008).

Silencing of *MKS1* gene in *Petunia* plants resulted in slight but not significant increase of the main stem length in comparison to control. As described in section above, the overexpression of the *MKS1* gene in investigated plants influences the growth habit and produces compact phenotype. That effect is most probably a result of increased SA levels in transgenic plants. However in plants with silenced *MKS1* gene the phenotype do not differ significantly from this of control plants. It might be assumed that low level of *MKS1* expression do not influence SA metabolism. It was reported by Andreasson et al. (2005), that *Arabidopsis* plants with silenced *MKS1* gene also did not exhibit phenotypic differences in comparison to the control plants.

MKS1-overexpressing *Petunia* plants tolerance to *Pseudomonas*

According to Andreasson et al. (2005) *Arabidopsis* 35S::*MKS1* transgenic plants exhibited elevated tolerance to the *Pseudomonas syringe* pv. tomato DC3000 bacteria. It is possibly attributed to the almost 4-fold increase in SA concentration and also elevated *PR1* transcript levels in examined mRNA. Rise of the SA concentration and *PR1* expression are the indicators of the SAR activation. The tolerance test in *MKS1*-transgenic *Petunia* plants showed that clones with 4 copies of the transgene exhibit higher resistance to *Pseudomonas* than those with one copy of the *MKS1* transgene. It was observed that the infection in clones with 4-copies of the transgene did not exceed symptoms more severe than third class symptoms, which are yellowish aureoles, while the non-transgenic control and clones with one copy of the transgene with time developed symptoms of 4^{th}, 5^{th} and 6^{th} class, which respectively are: yellowish leaf with a green edge, completely yellow curled leaves and

necrotic spots. It is possible that the expression level of *MKS1* in the *Petunia* clones with one copy of the transgene is lower than in those with four copies of the transgene. That might influence the lower SAR response in those clones, therefore lower tolerance to the pathogenic attack.

Conclusion and outlook

Conclusion and outlook

The strategy of influencing gibberellin or salicylic acid levels resulted in desired stem growth retardation. Unfortunately constitutive overexpression of both investigated genes resulted in unacceptable characteristics of transgenic plants. In case of both 35S::*GA$_2$ox* and 35S::*MKS1*-transgenic plants of *Kalanchoë* and *Petunia* significant delay of flowering was observed. This quality in potted or basket plants is unacceptable for producer and customers. Also the leaf morphology was altered in comparison to this in control plants. Although Lütken et al. (2011) claims that dark-green leaves add ornamental value for *Kalanchoë* this quality might be evaluated subjectively and it might not apply for other ornamental species. To avoid undesired effects of constitutive gene expression it was decided to overexpress *GA$_2$ox* gene with the stem-specific promoter (*dBI*). The resulted transgenic plants of *Kalanchoë* and *Petunia* exhibited dwarfed phenotypes, nevertheless, the leaf morphology was altered from those of the control plants. It was observed that the *dBI* promoter leads to expression in vascular tissue of the leaves and petioles. The leaves of transgenic *Kalanchoë* plants were round and convex and those of *Petunia* similar like in control plants but smaller. It would be desirable to continue with the observations of the reproductive growth in transgenic plants with stem-specific *GA$_2$ox* overexpression. Another strategy would be to change the promoter for one which would not lead to the expression in petioles or main vascular vein of the leaves in *Kalanchoë*.

In the last few months a new promising method of molecular manipulation of the genomes has gained popularity. Clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) is an efficient and precise genome editing with site-specific nucleases system which allows reverse genetics, genome engineering and targeted genome integration. In the light of the European regulations about genetically modified organisms CRISPR/Cas9 molecular breeding method gives hope for creating transgene-free mutated plants with the use of programmable nucleases, together with the transient expression of the nuclease components by agro-infiltration or viral vectors. This method allows to avoid one of the biggest GMO-related problems which is the random integration of the transgene which may lead to the unintended effects such as disrupted metabolism of the host plant or production of the toxic or allergenic compounds (Podevin et al., 2013). CRISPR/Cas9 gives possibility for external control of the targeted genes expression what is typically obtained with the introduction of the inducible or repressible

promoters. The disabled nucleases can still bind to their target DNA sequence and thus can be used for the regulation of expression. The dead Cas9, which is a catalytically inactive Cas9, is unable to cut DNA but it can still be recruited to specific DNA sequence by guide RNA. Provided that it is expressed as a fusion protein together with the transrepression or transactivation domain of a transcription factor the precise and reversible control of the transcription of the targeted genes may be achieved for example for transcription factors of genes which influence plant growth habit (Maeder et al., 2013, Gilbert et al., 2013). CRISPR/Cas9 enables to avoid the unwanted effects of regulatory elements and chromatin structure surrounding the transgene integration site influence. Also this method gives possibility to avoid silencing by giving the possibility to control transgene copy number, the presence of inverted repeats and truncated sequences. It would be desirable to continue the research on dwarfing genes and on stem-specific promoters with the use of CRISPR/Cas9 method to avoid among others the undesirable effects of the unspecific integration of the transgene in the genome.

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Acknowledgments

Acknowledgments

I would like to dedicate this thesis to my father who inspired me towards plant sciences and to my mother for all kinds of support and patience.

I would like to thank Prof. Dr. Margrethe Serek for her excellent support, care and guidance throughout my studies.

I would like to thank Dr. Heiko Mibus for introducing me to the molecular biology principles. Also I am sincerely grateful for the technical support in the laboratory and greenhouses to Mrs. Wiltrud von Oertzen, Mrs. Gisela Bölsing, Mrs. Annette Steding, Mr. Herbert Geyer and his crew.

I would like to thank Prof. em. Bjarne M. Stummann for his valuable advices in preparations of the research manuscripts.

And also I am very grateful to friends and colleagues at the Institute of Horticulture Production Systems for their reliability, inspiring discussions and exchange of experiences.

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2. 2005 – 2006 Exchange student (Erasmus program) at the University of Copenhagen, Horticulture, Copenhagen, Denmark (formerly named The Royal Veterinary and Agricultural University in Copenhagen), performance of the research project for M.Sc. Thesis: 'Germination, regeneration and transformation studies in *Campanula poscharskyana* and *Campanula portenschlagiana*'

3. 2006 – 2016 Ph.D. study (DAAD scholarship) at the Leibniz University of Hannover, Faculty of Natural Sciences, Institute of Horticulture Production Systems, Floriculture Germany on: 'Stem growth regulation by molecular breeding in *Kalanchoë blossfeldiana* and *Petunia hybrida'*

4. 2013 – 2014 Postgraduate study 'Business in Biotechnology' at the Jagiellonian University, Faculty of Biochemistry, Biophysics and Biotechnology and Jagiellonian Centre of Innovation, Kraków, Poland

5. 2011 – present, co-owner and manager at the Ornamental Plant Nursery, family business placed in Gaj – Kraków, Poland

Professional experiences:

1. 2003 – 2004 Realization of the project: 'The interference of hormones on *Euphorbia polychroma*'s root system, evaluation between three different medium' (oral presentation awarded at The International Agricultural Conference held in Krakow, Poland 2004);

2. 2003 – 2005 Taking part in a grant project on 'Evaluation of media components for establishing the most efficient *in vitro* regeneration protocol for *Rosa* 'Abraham derby' and 'Graham Thomas' cultivars', Horticulture Department, Ornamental Plants, University of Agriculture, Kraków, Poland

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Joanna M. Gargul's list of publications

International journals with referee system:

1. Gargul, J.M., H. Mibus and M. Serek, 2013. Constitutive overexpression of *Nicotiana* GA_2ox leads to compact phenotypes and delayed flowering in *Kalanchoë blossfeldiana* and *Petunia hybrida*. Plant Cell, Tissue and Organ Culture, 115: 407-418
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5. Gargul, J.M., H. Mibus and M. Serek, 2015. Molecular approach to plant growth regulation by constitutive overexpression of *Nicotiana* GA_2ox in *Kalanchoë blossfeldiana*. 1st International Symposium on Ornamentals in Africa, 09-13.09.2013, Naivasha, Kenya. Acta Horticulturae 1077: 165-171
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Entries in GenBank (NCBI, www.ncbi.nlm.nih.gov):
The entries contain details of submitted DNA sequences. The entries below are listed in the format: authors, year of publication in GB, accession No, [sequence-length] Title in GB.

8. Gargul, J.M., M. Serek and H. Mibus, 2013. KC782950.1 [676 bp] *Kalanchoë blossfeldiana* cultivar Molly putative protein phosphatase 2A (PP2) mRNA, partial cds

Abstracts:

9. Gargul J.M. and M. Piskornik 2004. The interference of hormones on *Euphorbia polychroma*'s root system, evaluation between three different medium' (oral presentation awarded at The International Agricultural Conference held in Krakow, Poland 2004) Book of Abstracts, p. 92, oral presentation.
10. Gargul, J.M., H. Mibus and M. Serek, 2010. Investigation of GA_2ox for growth control in *Petunia hybrida* and *Kalanchoë blossfeldiana* plants. Pflanzenbiotechnologie in Deutschland – Wo stehen wir? 13-15.09.2010. Hannover, Germany. Book of Abstracts, p. 17, oral presentation.
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12. Gargul, J.M., H. Mibus and M. Serek, 2014. Plant growth regulation by constitutive overexpression and silencing of *GA_2ox* in *Petunia hybrida*. Plant Signaling: Dynamic Properties 05-10.02.2014. Keystone, USA. Book of Abstracts, p. 54, abstract 2012, poster presentation
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14. Gargul, J., H. Mibus and M. Serek, 2015. Molecular breeding of *Petunia hybrida* 'Famous Lilac Dark Vein' for compact growth induction by *MKS1* and *GA_2ox* constitutive overexpression 28.06-02.07.2015, Melle, Belgium. Book of abstracts ISBN 9789040303678, Oral 02, page 26, oral presentation

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15. Gargul J.M., 2006. Germination, regeneration and transformation studies in *Campanula poscharskyana* and *Campanula portenschlagiana*. M.Sc. Thesis research performed at Section Horticulture, University of Copenhagen, Denmark, thesis defense at the Agriculture University of Kraków, Kraków, Poland. 109 pp.
16. Gargul J.M., 2016. Stem growth regulation by molecular breeding in *Kalanchoë blossfeldiana* and *Petunia hybrida*, Ph.D. Thesis, Leibniz University of Hannover, Faculty of Natural Sciences, Institute of Horticulture Production Systems, Floriculture Germany. 113 pp.