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ORIGINAL PAPER

Agroinfiltration of intact leaves as a method for the transient and stable transformation of saponin producing *Maesa lanceolata*

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Abstract A method has been developed to genetically transform the medicinal plant *Maesa lanceolata*. Initially, we tested conditions for transient expression of GFP-bearing constructs in agroinfiltrated leaves. Leaf tissues of *M. lanceolata* were infiltrated with *Agrobacterium tumefaciens* carrying a nuclear-targeted GFP construct to allow the quantification of the transformation efficiency. The number of transfected cells was depended on the bacterial density, bacterial strains, the co-cultivation time, and presence of acetosyringone. The transient transformation assay generated the highest ratio of transfected cells over non-transfected cells upon 5 days post-infiltration using *A. tumefaciens* strain LBA4404 at an $OD_{600} = 1.0$ in the presence of 100 μM acetosyringone and in the absence of a viral suppressor construct. In a second series of experiments we set up a stable transformation protocol that resulted in the regeneration of kanamycin-resistant plants expressing nuclear GFP. This transformation protocol will be used to introduce overexpression and RNAi constructs into *M. lanceolata* plants that may interfere with triterpenoid saponin biosynthesis.
Key message We have developed a transformation protocol for saponin producing *Maesa lanceolata*. Using the protocol reported here, now we are able to generate the tools for the modification of saponin production.

Keywords Agroinfiltration · Transient gene expression · Stable transformation · *Maesa lanceolata* · Saponin

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Introduction

Maesa lanceolata is used in African traditional medicine, whereby extracts of leaves and fruits are used for the treatment of various diseases including hepatitis, dysentery, skin diseases and neuropathies (Sindambiwe et al. 1996). Metabolic profiling has identified the presence of a mixture of triterpenoid saponin (maesasaponins) which have been tested for pharmaceutical activities (Muhammad et al. 2003; Sindambiwe et al. 1998; Tadesse et al. 2009). Specific subclasses of maesasaponins showed anti-angiogenic activity lacking haemolytic activity, which have a potential for the development of drugs for certain types of cancers (Apers et al. 2002).

Because maesaponins are highly complex structures, organic synthesis is not feasible for the further biological activity analysis and we therefore need to develop strategies that can modulate saponin biosynthesis in the plant. To begin with the identification of maesasaponin biosynthesis genes we have started to analyse *M. lanceolata* leaf mRNA sequences which will be reported elsewhere. As part of the characterization of candidate genes we have developed a transformation protocol for hairy root induction (Lambert et al. 2009). Although hairy root induction is perfectly suitable for the screening of different constructs and analysis of small samples of transgenic material, it may not be suitable for the expression of leaf-derived cDNA sequences. We therefore set up an *Agrobacterium tumefaciens*-mediated transformation protocol. Both a transient expression and a stable transformation method were developed. The transient expression system was optimized to allow the screening of candidate genes which are suspect to enhance saponin production or drive production towards one specific class of saponins.

Transient gene expression assays mediated by agroinfiltration have been successfully used as tool for rapid analysis of plant promoters and transcription in tobacco and rice (Yang et al. 2000; Zhang et al. 2012), isolation of disease resistance gene in potato (Bendahmane et al. 2000), and functional analysis of other genes in plants within a few days of infiltration (Bertazzon et al. 2012; Leckie and Neal Stewart 2011; Figueiredo et al. 2011; Hoffmann et al. 2006). The efficiency of transient gene expression is greatly influenced by the limitation of *A. tumefaciens* virulence and the physiological condition of the plants (Wroblewski et al. 2005). Therefore, an efficient method for transient gene expression and stable transformation in *Maesa* is critical to the study of gene function by overexpression or by gene silencing.

Notably, transient gene expression assays are readily applicable, do not require expensive supplies and equipment, and allow rapid screening of gene activity within leaf tissue (Wroblewski et al. 2005). In addition, it has been shown to work with great effectiveness in several model species, such as Arabidopsis, tobacco, tomato, potato, grapevine and rose (Tsuda et al. 2012; Yasmin and Debener 2010; Zottini et al. 2008; Kościańska et al. 2005; Bhaskar et al. 2009; Kim et al. 2009).

In this report, we describe the development of a transient expression system for *Agrobacterium*-mediated transformation of *M. lanceolata*. Under optimal transfection conditions, we have been able to develop a stable transformation method that takes advantage of the high transfection rates by directly transferring transfected leaf tissue onto regeneration and cultivation medium.

Materials and methods

Plant material and growth condition

M. lanceolata seeds were collected in Moshi, Tanzania by Frank Mbago (Department of Botany, University of Dar-Es-Salaam). The seeds were rinsed in 70 % (v/v) ethanol for 30 s and subsequently surface sterilized with a 7 % (v/v) solution of a commercial disinfectant (Haz-tabs; Guest Medical, Kent, UK) for 30 min. After three washes with distilled water, the seeds were placed on MS basal medium (Murashige and Skoog 1962) supplemented with 0.8 % (w/v) agar (Lab M plant tissue culture agar MC29, Amersham) and 3 % (w/v) sucrose (with pH 5.8). Seeds were germinated at 26 °C under a 16/8-h light/dark photoperiod (32.7 ± 3.23 μmol m^{-2} s^{-1}) provided by warm white fluorescent light (Osram, Germany). Seedling materials were then propagated through axillary shoot formation to produce clonal plant material in vitro. All the cultures were incubated in a growth chamber at the same condition as mentioned above.

Agrobacterium tumefaciens strain and expression vector

The pK7FWGF2 binary vector (Karimi et al. 2002) carrying an NLS::GFP (*Nuclear Localization Signal*, *Green Fluorescence Protein*) construct driven by 35S promoter was introduced to competent *A. tumefaciens* strain LBA4404 by heat shock transformation. The binary vector also contains an *npt*II kanamycin resistance gene for plant selection. The 35S::*p19* was made by inserting the PCR-amplified fragment of the *p19* into *Sma*I-linearized pBin61 (Voinnet et al. 2003).

Three days prior to infiltration, a single colony of *Agrobacterium* was started as preculture for 48 h on YEB medium (beef extract 5 g/L, yeast extract 1 g/L, peptone 5 g/L, sucrose 5 g/L and 2 mM $MgSO_4 \cdot 7H_2O$) supplemented with rifampicin 50 mg/L and spectinomycin 50 mg/L. Small aliquots of the preculture were transferred to fresh YEB medium with the same antibiotics and grown overnight with density of about $OD_{600} = 1.5$. The cultures were then centrifuged and the collected cells resuspended in infiltration medium (10 mM $MgCl_2$, 10 mM MES pH 5.6, and 100 μM acetosyringone) and OD_{600} was adjusted to 1.0. The suspension was then incubated for 2 h at room temperature prior to infiltration. The virulence of the *Agrobacterium* was modulated by supplementing the bacterial culture medium with 0, 50, 100, 150 and 200 μM acetosyringone.

Agroinfiltration and immersion of *M. lanceolata* leaves

M. lanceolata was routinely acclimatized in the greenhouse from in vitro culture, 3 weeks prior to agroinfiltration. Agroinfiltration was performed as previously described (Voinnet et al. 2003), with some modifications. The 2nd to 5th mature leaves counting from the apex were used for infiltration. Approximately 0.5 mL of the *Agrobacterium* cell suspension was injected at the abaxial side using a 5 mL syringe. Two or three injections were sufficient to infiltrate the cell suspension throughout each leaf. After infiltration, the plants were placed back under the same growth conditions. Co-infiltration with *p19* was done by mixing the bacterial cultures with final OD_{600} adjusted to 1.0.

For the immersion technique, leaf discs were immersed for 10 min in an *Agrobacterium* culture of OD_{600} 1.0 resuspended in infiltration medium. Treated leaf discs were blotted dry on sterile filter paper and placed adaxial side down on shoot induction medium without antibiotics. After 5 days of co-cultivation, leaf discs were transferred to

shoot induction medium (SIM) containing MS medium (Murashige and Skoog 1962) supplemented with 22.2 μM TDZ, 1.35 μM NAA, 3 % sucrose, and 0.8 % agar to induce adventitious shoot formation. 500 mg/L cefotaxime and 100 mg/L kanamycin were also added to SIM to select the transgenic shoots.

Evaluation of transient gene expression

Pieces of leaf were randomly cut from injected areas and mounted on slides for microscopic observation. To track the GFP expression, an inverted Olympus IX81 microscope with CellM^{TM} software (Olympus) was used, equipped with an XM-10 (Olympus) camera. The number of fluorescence nuclei in each image (1,373 × 1,038 pixel size) was calculated using ImageJ software (Rasband 1997–2009). Only those fluorescence nuclei larger than 10 pixels were used for quantification.

After evaluation of GFP expression, agroinfiltrated leaves of *M. lanceolata* were cut and fixated with ethanol:acetic acid buffer (3:1) overnight. Subsequently, leaves were washed with water to remove the remaining fixative solution. For nuclei staining, leaves were incubated with a 2 ng/μL DAPI (4′,6′ diamino-2-phenylindole) solution for 30 min followed by washing with water to reduce background interference and mounted on slides for microscopic observation. Frequency of transient transformation was scored by comparing the number of GFP-nuclei with the number of DAPI-stained nuclei, multiplied by 100 % [(n GFP/n DAPI) × 100].

Stable transformation from transient gene assay

Effect of cefotaxime

Cefotaxime was used to prevent the over growth of *Agrobacterium*. To determine the optimum cefotaxime concentration for maximum shoot regeneration, we incubated leaf discs on shoot induction medium containing different concentrations of cefotaxime (0, 125, 250 and 500 mg/L). The cultures were incubated with same growth conditions as mentioned above.

In vitro establishment of infiltrated leaves

For stable transformation, the infiltrated leaves were sterilized for 30 min with 10 % (v/v) solution of Haz-tab solution and a drop of Dreft^{TM} detergent (Procter and Gamble, UK) and rinsed three times with distilled water. Sterilized explants were placed on shoot induction medium supplemented with 500 mg/L cefotaxime and 100 mg/L kanamycin to select the transgenic plants. Multiple shoots were induced through adventitious shoot regeneration as previously described (Faizal et al. 2011).

PCR analysis

Leaf samples of approximately 100 mg of in vitro regenerated shoots were collected for genomic DNA isolation. The collected samples were frozen immediately in liquid nitrogen and genomic DNA was extracted using DNeasy Plant Mini Kit (Qiagen). For PCR analysis, primers were designed for the amplification of the *npt*II kanamycin resistance gene and *GFP* gene. The 412-bp fragment of *npt*II was amplified using oligo-nucleotide primers of 5′-GATGTTTCGCTTGGTGGTC-3′ and 5′-GAACAAG ATGGATTGCACGC-3′. The 800 bp of *GFP* was amplified using oligo-nucleotide primers of 5′-TAGTCGA CCTGCAGGCGGC-3′ and 5′-TTTCTCGAGTTACTTG TACAGCTCGTCCATGCC-3′. *vir*G primers (5′-GCCGG GGCGAGACCATAGG-3′ and 5′-CGCACGCGCAAGG CAACC-3′), which amplify the 606-bp fragment of *vir*G gene (Schaart et al. 2004) and *vir*C primers (5′-GG CGGGCGCGCCGAAAGGAAAACCT-3′ and 5′-AAGA ACGCGGAATGTTGCATCTTAC-3′), which amplify the 414 bp of *vir*C gene (Suzaki et al. 2004) of *A. tumefaciens* were used to determine the presence of *A. tumefaciens*. The following cycling conditions were tested: 95 °C for 2 min; 30 cycles at 95 °C during 30 s, 54 °C during 30 s, 72 °C during 30 s for *npt*II and *vir*C or 1 min for *GFP* and *vir*G; followed by a final incubation at 72 °C for 5 min. The amplified products were separated on 0.8 % agarose gel electrophoresis and visualized with ethidium bromide.

Statistical analysis

Data were analysed using one-way ANOVA and the comparisons of the mean GFP expression was contrasted using Duncan's multiple range test. All statistical analyses were performed at the level of *P* value less than 0.05 using SPSS 18.0 (SPSS Inc. USA).

Results

Transient expression upon agroinfiltration of intact leaves

The level of transient expression in an agroinfiltration system depends on the efficiency of transformation and the transcriptional and translational activity of the monitored marker. In preliminary experiments, we tested seven different *Agrobacterium tumefaciens* strains (C58, EHA101, EHA105, LBA4404, GV3301, GV2260 and pMP90), and found that LBA4404 resulted in consistently higher

transformations than the other strains (data not shown). The nuclear-targeted GFP reporter (NLS-GFP) carried by *Agrobacterium tumefaciens* strain LBA4404 was highly expressed in transformed leaf cells and allowed rapid and reliable identification of cells that were transformed and expressed the nuclear GFP in infiltrated leaves (Fig. 1). The transient transformation assay was optimized by evaluating the effects of bacterial density, the co-cultivation time, the presence of acetosyringone and the presence of a viral silencing suppressor construct.

The transformation efficiencies were determined by dividing the average of GFP fluorescent nuclei (Fig. 2a) by the average number of DAPI-stained nuclei observed within a transfected area of the same leaf blade (Fig. 2b).

The bacterial density

Bacterial cell density of *Agrobacterium* suspensions has been shown to be an important factor in transient expression in several plants (Wroblewski et al. 2005; Bhaskar et al. 2009). In Arabidopsis, cell suspensions with a cell density less than $OD_{600} = 0.1$ often result in low expression and those with cell densities above $OD_{600} = 1.0$ result in tissue yellowing or wilting (Wroblewski et al. 2005). We tested different cell densities of *A. tumefaciens* LBA4404 of OD_{600} 0.1, 0.3, 0.5, 0.8, 1.0, 1.3, and 1.6. As shown in Fig. 3a, the transient expression level initially increased with the increasing density of *Agrobacterium*. It reached the maximum transient expression level at OD_{600} of 1.0 and then decreased at OD_{600} higher than 1.0.

The co-cultivation time

In the leaf blades we infiltrated, we did not observe plant cell division or substantial leaf expansion during the period of transfection (data not shown). The cells showed transient NLS::GFP expression from the second day post-infiltration (dpi) and the number of cells expressing GFP increased to a maximum at 5 dpi. Afterwards the number of cells expressing GFP as well as the intensity of fluorescence decreased significantly (Fig. 3b).

The presence of acetosyringone

We also investigated the effect of acetosyringone since it has been reported to influence transformation efficiency

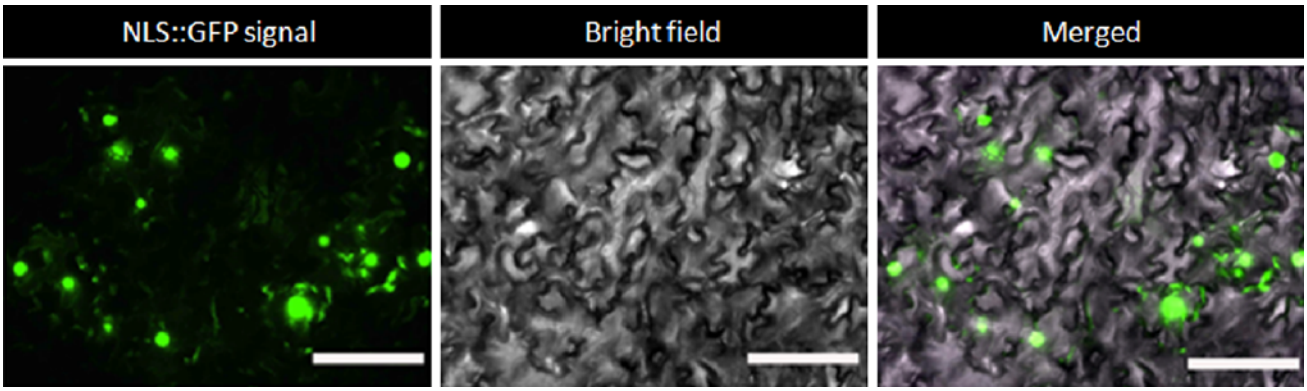

Fig. 1 Microscopy images showing the nuclear-targeted GFP fluorescence from agroinfiltrated leaf tissue. *Bar* 100 μm

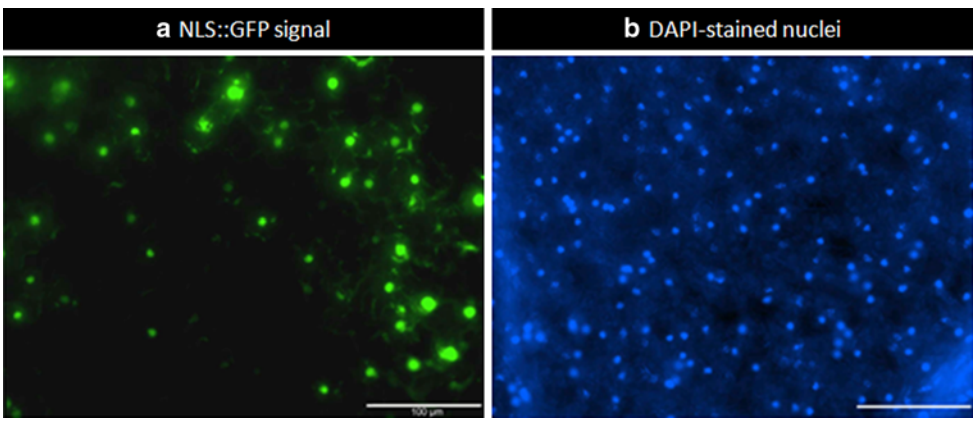

Fig. 2 Microscopy analysis of transfected *M. lanceolata* leaf blades. **a** NLS-GFP fluorescence showing fluorescent nuclei, **b** fluorescence from DAPI-stained nuclei at a similar region of the transfected leaf blade. Images were taken with the same magnification, *bar* 100 μm

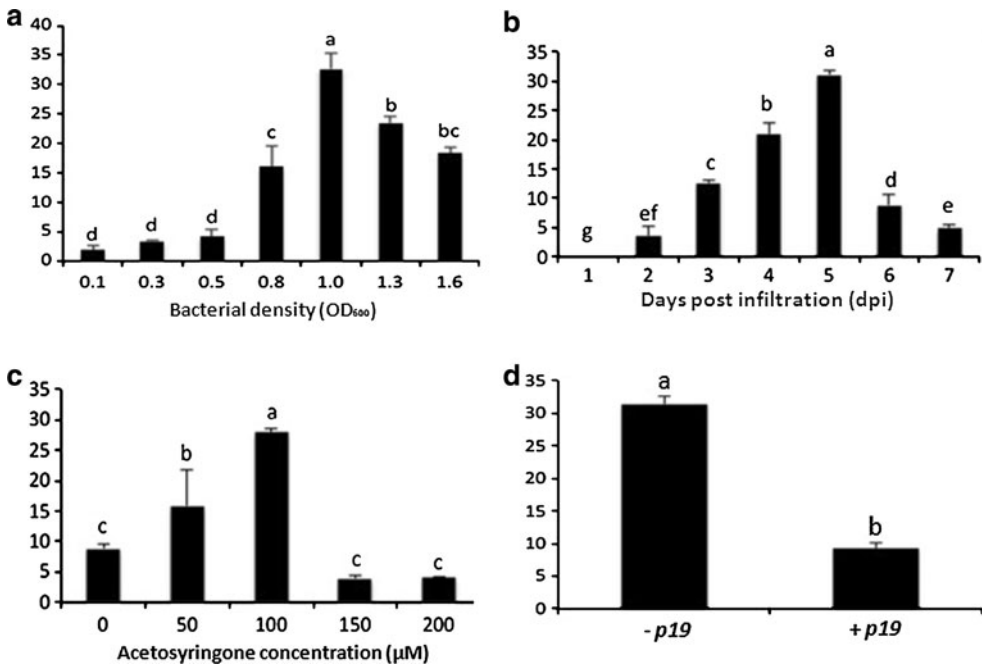

Fig. 3 Frequency of transient GFP expression for the effect of bacterial density (**a**), days post-infiltration (**b**), the presence of acetosyringone (**c**) and co-transfection with *p19* (**d**). The *y* axis indicates the mean frequency of transient GFP expression of each factor. The frequency data were generated with at least 5 replicates and 3 independent repetitions. *Different letters* above each column indicate significant differences between the mean values at $P < 0.05$

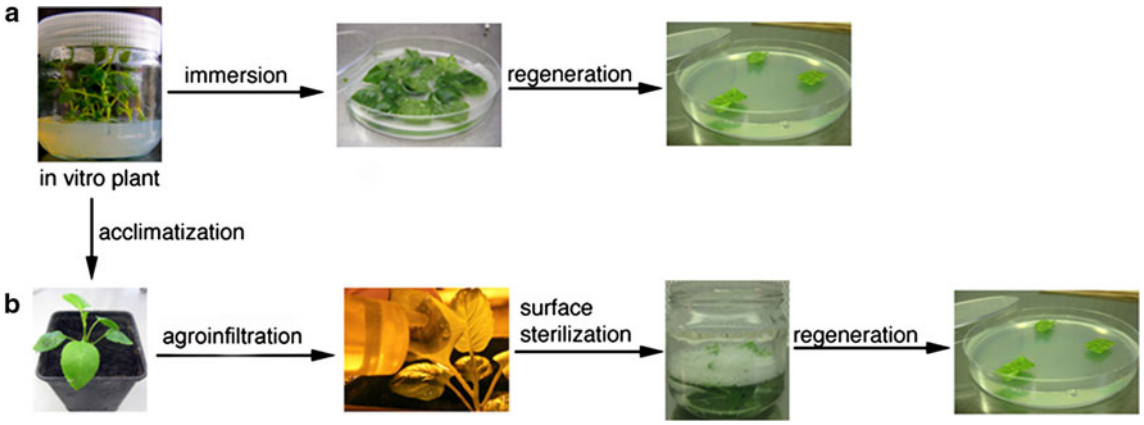

Fig. 4 Schematic diagram of *A. tumefaciens*-mediated transformation (**a**) immersion and (**b**) agroinfiltration method

and transient gene expression in many plant species (Godwin et al. 1991; Kapila et al. 1997). Acetosyringone was added to the infiltration medium at different concentrations. The addition of 50 µM of acetosyringone doubled the transformation efficiency. Furthermore, the transformation efficiency achieved its maximum level when the concentration of acetosyringone was increased to 100 µM. Concentrations higher than 100 µM acetosyringone had a negative effect on the transformation efficiency (Fig. 3c).

Co-infiltration with a viral silencing suppressor

Dhillon and co-workers reported that co-introduction of plant viral suppressors of gene silencing *HCPro* (from *Tobacco etch virus*), *p19* (from *Tomato bushy stunt virus)*, *γb* (from *Barley stripe mosaic virus*) and *p21* (from *Beet yellows virus*) with GFP on a separate plasmid lead to an almost twofold increase in initial GFP expression levels in *Phaseolus lunatus* (Lima bean) cotyledons (Dhillon et al. 2009). Therefore, we also co-introduced one of the silencing suppressors, *p19* with the GFP gene on a separate plasmid via agroinfiltration and evaluated the effect on transient GFP expression in *M. lanceolata*. In the case that the transient gene assay would be limited by post-transcriptional gene silencing (PTGS), the co-infiltration with *p19* could enhance transfection rates. However, compared to the GFP expression plasmid alone, co-infiltration of the NLS::GFP with *p19* resulted in significantly lower transfection and GFP expression (Fig. 3d).

Stable transformation of *M. lanceolata*

Initial attempts to transform *M. lanceolata* using leaf discs immersed with *Agrobacterium* cultures did not result in transgenic plants. Also, an inspection of the immersed tissue showed that very few of the cells at the cutting edges were transfected. Having established an optimized transient transformation assay, we decided to use infiltrated leaf discs for generating stable *M. lanceolata* transformants. To compare the classic leaf disc immersion technique with the infiltrated leaves we performed experiments in parallel. A schematic view of the two approaches is shown in Fig. 4. The dose of antibiotics required for selection was tested by incubating the leaf discs on shoot induction medium containing different concentration of cefotaxime and kanamycin (Table 1). Kanamycin at 25 mg/L completely inhibited shoot regeneration from leaf explants following a protocol previously described (Faizal et al. 2011). Because leaf explants showed resistance to higher kanamycin concentrations, we used 100 mg/L. At this concentration, kanamycin had a necrotic effect on axillary propagated shoots. This concentration was effective to select stably transformed plants without lethality.

Table 1 The effect of cefotaxime and kanamycin on adventitious shoot regeneration induced from *M. lanceolata* leaf explants incubated for 8 weeks on shoot induction medium (SIM)

Antibiotic (mg/L)		No. of shoot/explant ± SE
Cefotaxime	Kanamycin	
–	–	3.3 ± 0.7^{b}
125	–	2.8 ± 0.8^{b}
250	–	8.7 ± 1.4^{a}
500	–	10.1 ± 1.3^{a}
–	25	0.00
–	50	0.00
–	75	0.00
–	100	0.00

Each Petri dish with four explants was considered as one replicate. The presented experiments comprise at least 5 replicates with 3 repetitions. *Different letters* indicate significant differences between mean values at $P < 0.05$ within each treatment

The impact of cefotaxime on regeneration capacity was also analysed. Cefotaxime is usually applied to stop growth and eliminate remaining *Agrobacteria*. The influence of cefotaxime on shoot induction was examined by incubating leaf explants on shoot induction medium containing 0, 125, 250, and 500 mg/L cefotaxime. Cefotaxime at 250 and 500 mg/L had a stimulatory effect and significantly increased the number of adventitious shoots emerging on the leaf explants compared to control leaf explants. Therefore, for the subsequent experiments we used 100 mg/L kanamycin and 250 mg/L cefotaxime for transgenic selection.

To compare with the classical transformation method of *Agrobacterium* immersion, we submerged leaf discs

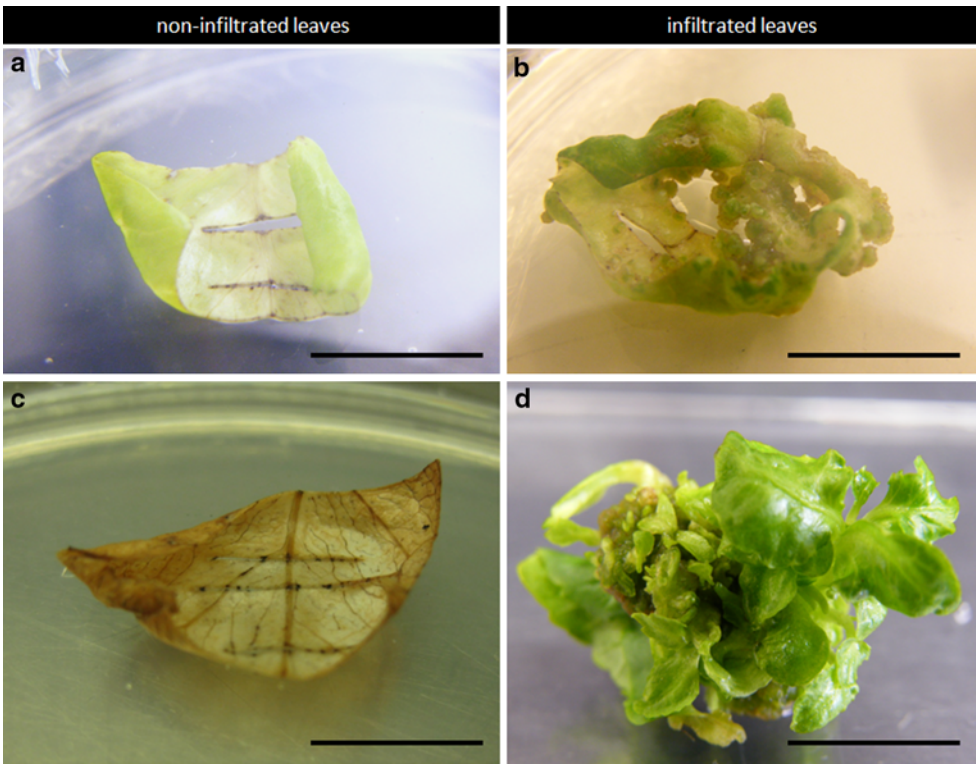

Fig. 5 Adventitious shoot regeneration of *M. lanceolata* starting with leaf explants (**a**, **b**) 4 weeks after incubation and (**c**, **d**) 8 weeks after incubation on selective medium. Control leaf discs did not show signs of callus formation and lost chlorophyll over time (**a**, **c**). Agroinfiltrated leaves and the transgenic shoot regeneration from infiltrated leaves (**b**, **d**). *Bar* 1 cm

(3 repeats, every repeat with at least 30 leaf discs) in *Agrobacterium* suspension. The immersion of leaf discs did not result in the formation of kanamycin resistant shoots. However, agroinfiltrated leaf discs were very efficient at producing kanamycin-resistant shoots (Fig. 5). We isolated 62 independent transgenic lines that were regenerated from 25 leaf explants by leaf disc agroinfiltration. The adventitiously formed shoots spontaneously generated roots in hormone-free MS medium. The rooting success was comparable to the non-transformed adventitious shoots as previously reported (Faizal et al. 2011). The plants were transferred to soil and developed into vigorously growing plants under greenhouse conditions.

PCR analysis

To determine whether the transgenic shoots carried NLS::GFP-containing T-DNA constructs, we analysed eight regenerated shoots by PCR. A comparison was made between genomic DNA isolated from the transformed and non-transformed shoots. pK7FWGF2 plasmid was used as a positive control. Primers were designed for the amplification of the *npt*II kanamycin resistance gene. PCR reactions using DNA from non-transformed shoots produced two fragments but these did not correspond in size to what is predicted from the *npt*II sequence. The same fragments were also detected in PCR reactions with DNA from candidate transgenic shoots. However, the samples from candidate transgenic shoots generated a PCR fragment of about 400 bp that corresponds to the *npt*II amplification product for the selected primers (Fig. 6a). We therefore conclude that the *npt*II primers generated unspecific as well as *npt*II-derived PCR products. To evaluate the presence of GFP sequences in transgenic shoots, PCR reactions using GFP-specific primers were conducted. All the candidate transgenic shoots generated a PCR fragment of 800 bp which corresponds to the predicted *GFP* PCR product (Fig. 6b). To determine whether extracellular *A. tumefaciens* proliferation would be the template source for GFP PCR amplification, we performed PCR analysis with primers that amplify the genomic *Agrobacterium virG* and *virC* genes. The analysis showed that the predicted PCR product is obtained with *Agrobacterium* DNA extracts as template, but not when plant extracts were used (Fig. 6c, d). We therefore conclude that there was no *Agrobacterium* contamination in the extract of transgenic plants.

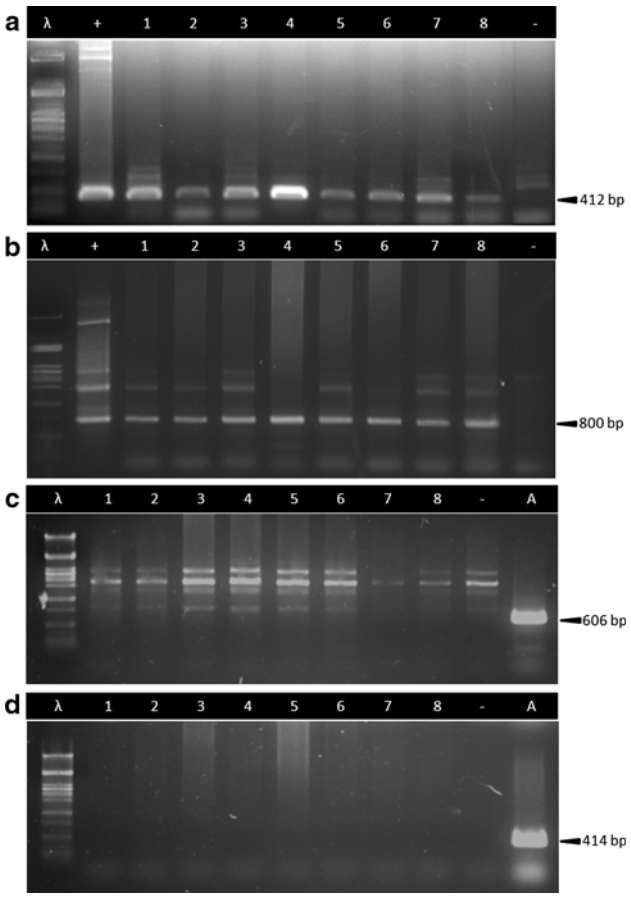

Fig. 6 PCR analysis of plants regenerated on kanamycin-containing medium. Genomic DNA was isolated from 8 individual transformation experiments. The PCR products of amplified 412 bp (**a**), 800 bp (**b**), 606 bp (**c**), and 414 bp (**d**) fragments corresponding to the nptII kanamycin resistance, GFP, *vir*G and *vir*C genes, respectively. *λ* Lambda DNA/*Pst*I standard, + positive control (plasmid pK7FWGF2), *lanes 1–8* transformed plants, − negative control (untransformed plant) and *A A. tumefaciens*

Discussion

Transient gene expression

It is known that some *Agrobacterium* strains are more virulent than others, depending on the target plant species. For example, it has been indicated that the C58 strain is most suitable for the transient expression assay in lettuce, tobacco and Arabidopsis (Wroblewski et al. 2005). Previous reports suggest that variations in transformation efficiency are caused by several factors including differences in ability of the bacterial cells to attach to plant cells, and differences in *vir* region and the chromosomal background (Nam et al. 1997; Chen et al. 2010). Because these factors are not likely influenced by different incubation conditions all subsequent experiments were performed using the LBA4404 strain. Much less is known about the host components which also affect the recognition process, the transfer of T-DNA, and the integration of T-DNA (Kim et al. 2009). To minimize variations in transformation efficiencies, we used in vitro clonally propagated shoot material.

The density of *Agrobacterium* suspension had a significant effect on GFP transient expression. The results

indicate that the GFP expression occurred at OD_{600} values between 0.1 and 1.6 with maximum expression level at bacterial density of OD 1.0. This is similar to findings in grapevine (Santos-Rosa et al. 2008) and Arabidopsis (Kim et al. 2009) where densities as low as OD 0.1 induced at least a weak expression. In contrast, the transient gene expression in rose petals was only observed for bacterial densities greater than 0.5 (Yasmin and Debener 2010). Necrosis and withering have been reported in tobacco and tomato to depend on the bacterial density (Wroblewski et al. 2005). In our study, however, necrosis and withering of *M. lanceolata* leaves did not occur in any of the tested conditions.

Acetosyringone is already known for its ability to activate virulence genes of *Agrobacterium* necessary to transfer T-DNA (Gelvin 2003). There are several reports that show acetosyringone significantly improves the transient expression of foreign genes (Ozawa and Takaiwa 2010; Kapila et al. 1997; Subramanyam et al. 2011). In contrast to these studies, the addition of acetosyringone did not cause transient transformation in lettuce, tomato, Arabidopsis (Wroblewski et al. 2005), and rose petals (Yasmin and Debener 2010). The presence of acetosyringone in agroinfiltration buffer was essential for maximum transient expression in *M. lanceolata*. Our results showed that the addition of 100 μM of acetosyringone increased the transient expression by approximately threefold compared to those without additional acetosyringone.

RNA silencing or PTGS has also been considered to play a major role in the post-introduction gene expression decline in transient expression (Dhillon et al. 2009; Voinnet et al. 2003). Therefore, we evaluated one of the suppressors of plant gene silencing, *p19* to combat the PTGS response from *M. lanceolata*. *p19* is a suppressor isolated from *Tomato bushy stunt virus* (TBSV) and inhibits the formation of the initiator RNA-inducing silencing complex (RISC) either by preventing the unwinding of duplex siRNAs or by sequestering the duplex siRNAs (Lakatos et al. 2004). However, we consistently have approximately a threefold decrease in frequency of transient expression when *p19* was co-transfected. Wroblewski and co-workers reported that the co-introduction of viral silencing suppressor clearly resulted in stronger transient gene expression *Nicotiana benthamiana* (Wroblewski et al. 2005). However, none of tested silencing suppressors had an observable effect on transient GFP or GUS expression in lettuce and Arabidopsis (Wroblewski et al. 2005). Therefore, the ability of silencing suppressor like *p19* to prevent PTGS of transiently expresses genes appears to be specific on the plant genotype.

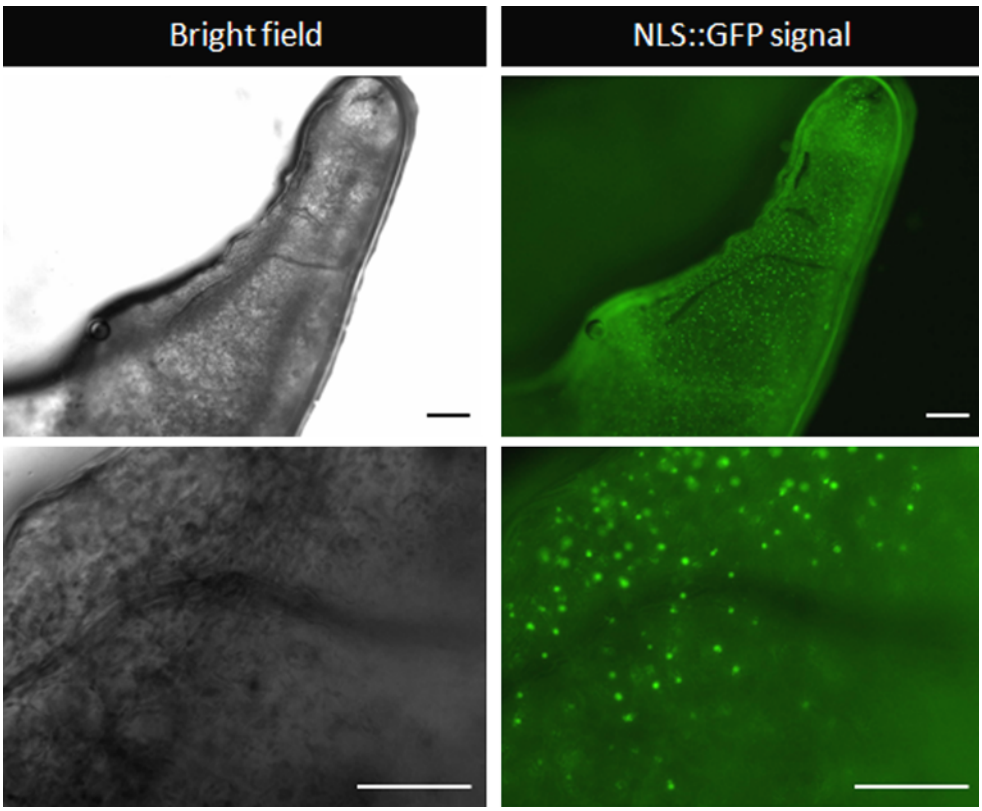

Fig. 7 Microscopy images showing the nuclear-targeted GFP of the in vitro regenerated shoots. *Bar* 100 μm

Stable transformation

The optimized transient *A. tumefaciens*-mediated transformation technique for *M. lanceolata* involves a 5-day co-cultivation with the *Agrobacterium* strain LBA4404 and OD_{600} of 1.0 supplemented with 100 μL acetosyringone. In the absence of successful transformation with submerged leaf tissue, we dissected the transfected leaf material and used an established in vitro regeneration protocol of *Maesa* (Faizal et al. 2011) for the regeneration of transgenic tissues of *M. lanceolata*. Shoot regeneration was stimulated in the presence of 250–500 mg/L cefotaxime. The same effect was reported in *Eucalyptus tereticornis* where the addition of 500 mg/L cefotaxime increased the number of shoot regeneration (Aggarwal et al. 2010). The increasing number of regenerated shoots could be due to changes in the endogenous levels of plant growth regulators (PGRs). It was reported that the regulatory activities of cefotaxime could be due to its interference with the metabolism of PGRs (Plus et al. 1993). In our study, however, the agroinfiltrated leaves produced the same average number of shoots as the control or about 3 shoots per leaf explant. This showed that the number of regenerated shoots is limited by kanamycin, which only allowed the transformed cells to regenerate.

In the current study, we compared immersion of *M. lanceolata* leaf explants in *Agrobacterium* suspensions with agroinfiltrated material. Leaf discs immersed with *Agrobacterium* suspension did not show shoot regeneration and lost chlorophyll over time on SIM supplemented with 100 mg/L kanamycin. The same result was obtained on control/non-infiltrated leaves. In contrast, a number of plant cells could be regenerated and stably transformed by agroinfiltration. Similar to our findings, using agroinfiltration in the creation of marker-free tobacco plants was also more successful than using the immersion technique (Jia et al. 2007; Kopertekh and Schiemann 2005). We speculate that agroinfiltration has an advantage over immersion because in agroinfiltrated tissue more plant cells are accessible to *Agrobacterium* adherence and interaction. It will be of interest to see if the technique also promotes transformation effectiveness in other plant species.

The stability of transformed *M. lanceolata* was easily visualized because the majority of cells showed nuclear-targeted GFP expression (Fig. 7) and this GFP expression was stable even in plants grown in the greenhouse (data not shown). Further confirmation of T-DNA integration was obtained by PCR analysis for eight independent shoot lines. Ongoing transformation experiments have shown that regenerated shoots are invariably transgenic (data not shown). The *vir*G and *vir*C PCR analysis supports the absence of *Agrobacterium* contamination affirming that the NLS-GFP-expressing shoots are genuine stable transformants.

In conclusion, the protocol reported here for *Agrobacterium*-mediated transformation of *M. lanceolata* is very efficient for high frequency transient gene expression of leaf tissues. In this study, we also proved that agroinfiltration can be used as an alternative approach to regenerate stably transformed plants. The stably transformed plants could be obtained in 3–4 months. On this basis, now we have a technique for engineering saponin biosynthesis from *M. lanceolata*. This technique can also be applied to other plant species that are permissive for agroinfiltration.

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