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Abstract

Lavandin (*Lavandula* x *intermedia* Emeric ex Loiseleur) is an aromatic plant, the essential oil of which is widely used in the perfume, cosmetic, flavouring and pharmaceutical industries. The qualitative or quantitative modification of its terpenes-containing essential oil by genetic engineering could have important scientific and commercial applications. In this study, we report the first *Agrobacterium tumefaciens*-mediated gene transfer into lavandin. The transformation protocol was optimized by lengthening precultivation and cocultivation periods and by testing five different bacterial strains. We obtained transformed callus lines at a frequency of 40–70% with strains AGL1/GI, EHA105/GI and C58/GI. Transgenic shoots were regenerated from these kanamycin resistant calli and rooted on selective medium with 150 mg l^{-1} kanamycin. The final percentage of transgenic plants obtained varied from 3 to 9%, according to the *Agrobacterium* strain used, within 6 months of culture. The presence of the introduced β-glucuronidase and neomycin phosphotransferase II genes was shown both by PCR and Southern blot analysis. Transgene expression was investigated using histoenzymatic β-glucuronidase assays, leaf callus assays and RT-PCR. Results showed that both β-glucuronidase and neomycin phosphotransferase II genes were expressed at a high level in at least 41% of the transgenic plants regenerated. This efficient transformation strategy could be used to modify some genetic traits of lavandin (flower colour, pathogens resistance) and to study the biosynthesis of the major monoterpene components of its essential oil (linalool, linalyl acetate, camphor and 1,8-cineole).

Introduction

Lavandin (*Lavandula* x *intermedia* Emeric ex Loiseleur), a spontaneous hybrid originating from the entomophilous crossing of lavender (*L. angustifolia* Mill.) and spike lavender (*L. latifolia* Medic.), has a high economic value. Its essential oil is widely used in the perfume, cosmetic, flavouring and pharmaceutical industries instead of the more expensive oil of lavender (Segura & Calvo, 1991). Lavandin also shows a large geographic plasticity, rapid growth and a high yield of monoterpenes, the major components of its essential oil. For these multiple reasons, its cultivation has been widely developed and has largely replaced that of lavender. Monoterpene composition of lavandin essential oils has been exhaustively documented and four main compounds (linalool, linalyl acetate, camphor and 1,8- cineole) have been identified (Lawrence, 1994; Boelens et al., 1995). Although the biochemical steps leading to synthesis of various monoterpenes have been extensively studied in several species such as *Mentha* x *piperita*, *M. spicata*, *Salvia officinalis*, *Clarkia breweri* (McGarvey & Croteau, 1995; McCaskill & Croteau, 1997), the biosynthesis pathway of monoterpenes is still unknown in lavandin.

Genetic transformation could aid in both understanding and modifying the pathways of monoterpene synthesis in lavandin. Such studies have been reported

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in other species. For example, the function of the gene pTOM5 involved in the carotenoid biosynthesis in tomato, was elucidated using a transgene strategy (Bird et al., 1991). In the same way, in tobacco, the overexpression of genes implicated in the biochemical steps leading to terpene production (*e.g.* the genes encoding 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 or farnesyldiphosphate synthase) was correlated to an accumulation of end-products of the pathway, such as sterols and carotenoids (Schaller et al., 1995; Daudonnet et al., 1997). Recently, progress in analytical biochemistry has allowed the characterization and purification of several enzymes in the monoterpene pathway and some corresponding cDNAs have been cloned, *e.g.* 4*S*-limonene synthase from *M. spicata* (Colby et al., 1993) and *Abies grandis* (Bohlmann et al., 1997), *S*-linalol synthase from *C. breweri* (Dudareva et al., 1996), sabinene synthase and 1,8-cineole synthase from *S. officinalis* (Wise et al., 1998) and myrcene synthase and pinene synthase from *A. grandis* (Bohlmann et al., 1997). As some of these genes could be present in the nuclear genome of lavandin, the modification of their expression level by ectopic overexpression, antisense strategies and / or tissue specific expression could provide a better understanding of the monoterpene synthesis pathway of lavandin. However, to date, genetic transformation of this species has not been reported.

A prerequisite for such genetic transformation is the availability of an efficient regeneration system. Recently, *in vitro* plantlet regeneration has been successfully obtained using lavandin leaves (Dronne et al., 1999). In this study, we investigated the suitability of this regeneration protocol for *Agrobacterium tumefaciens*-mediated gene transfer using the *gus* and *nptII* genes. In addition, we present, for the first time in lavandin, a regeneration method for transgenic plants which could be a suitable experimental tool for the bioengineering of essential oil composition.

Materials and methods

Plant material

In these experiments, the cultivar 'Grosso 2' of lavandin (*Lavandula* x *intermedia* Emeric ex Loiseleur), the genotype most widely cultivated in France, was used. Plants were maintained in a greenhouse at 25 ± 2°C under a 16 h photoperiod (lamps Mazda MAIH 400, 550 μmol m^{-2} s^{-1}). Young fully expanded leaves taken from the first and second node of 4-year old plants were surface-sterilized by soaking for 20 s in 70% (v/v) ethanol and then 15 min in 5% (w/v) sodium hypochlorite containing a few drops of Tween 80. After three rinses in sterile water, leaves were cut into sections 5–7 mm in length and placed on culture medium.

Bacterial strains

Five *Agrobacterium tumefaciens* strains with different chromosomal backgrounds and different disarmed virulence plasmids were used: LBA4404 (pAL4404) (Hoekema et al., 1983), C58 (pMP90) (Koncz & Schell, 1986), C58 (pGV2260) (Deblaere et al., 1985), EHA105 (pEHA105) (Hood et al., 1993) and AGL1 (pAGL1) (Lazo *et al.*, 1991). The binary plasmids in these strains harboured both the selection marker gene *nptII* and the reporter gene *gus*, but they were derived from various origins (Table 1). The LBA4404 (pAL4404), C58 (pMP90) and C58 (pGV2260) strains contained the p35S *gus* plasmid (Vancanneyt et al., 1990), while EHA105 (pEHA105) and AGL1 (pAGL1) strains contained respectively the pMOG410 plasmid (Figure 1) (Hood et al., 1993; kindly provided by Mogen International, The Netherlands) and the pB+GIN plasmid (constructed and provided by L. Jouanin, INRA Versailles, France). These strains were named LBA4404/GI, C58/GI, GV2260/GI, EHA105/GI and AGL1/GI respectively.

Bacterial cultures were grown overnight at 24°C on a rotary shaker (120 r.p.m.) in 40 ml LB (Sigma, USA), with appropriate antibiotics and 50 μM acetosyringone (Aldrich, Germany). When an OD 600 nm of 0.5–0.7 was reached, bacterial suspensions were centrifuged (3000 × *g*, 15 min). The pellets were washed twice and then diluted 10-fold in liquid callogenesis medium. These suspensions were used for cocultivation with the plant material.

Transformation procedure and plantlet regeneration

Transformation and regeneration of plantlets were achieved using four successive culture media described previously by Dronne et al. (1999). The basal medium culture (BM) consisted of MS salts (Murashige & Skoog, 1962) supplemented with Morel & Wetmore (1951) vitamins and 20 g l^{-1} sucrose. It was solidified with either 0.15% (w/v) phytagel (Sigma, USA) for callogenesis and caulogenesis steps or 0.7% (w/v) agar (Sigma, USA) for shoot elongation and

Table 1. Chromosomal background, disarmed virulence plasmid and binary plasmid of each *Agrobacterium* strain used for transformation of lavandin

Agrobacterium tumefaciens strains	Shortered name	Chromosomal background	Disarmed virulence plasmid	Binary plasmid
LBA4404 (pAL4404)	LBA4404/GI	Ach5	Octopine	p35S *gus*
C58(pMP90)	C58/GI	C58	Nopaline	p35S *gus*
C58(pGV2260)	GV2260/GI	C58	Octopine	p35S *gus*
EHA105 (pEHA105)	EHA105/GI	C58	L,L Succinamopine	pMOG410
AGL1(pAGL1)	AGL1/GI	C58	L,L Succinamopine	pB+GIN

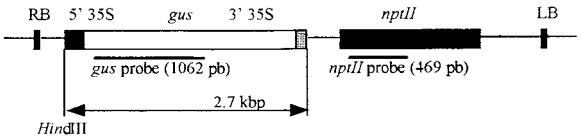

Figure 1. Schematic representation of the T-DNA of pMOG410 plasmid, showing the restriction sites for *Eco*RI, *Hin*dIII, the relative size of *gus* and *nptII* probes and the expected hybridization products.

rooting steps. The pH was adjusted to 5.8 prior to autoclaving (115°C, 15 min).

Leaf explants were first precultured for either 2 or 8 days on callogenesis medium (BM supplemented with 9 μM BAP and 4.5 μM NAA). Leaves were then immersed in the bacterial suspension and shaken at 40 r.p.m. for 30 min before being blotted dry on sterile filter paper and cocultivated on callogenesis medium for 2 or 4 days. Leaf explants were then partially decontaminated after rinsing 3 times for 10 min in liquid callogenesis medium supplemented with 400 mg l^{-1} Augmentin® (Smithkline Beecham, France) and transferred onto callogenesis medium supplemented with 70 mg l^{-1} kanamycin monosulfate (Sigma, USA) and 400 mg l^{-1} Augmentin® for 6 weeks. Calli obtained were then transferred onto caulogenesis medium (BM with 18 μM BAP, 70 mg l^{-1} kanamycin and 400 mg l^{-1} Augmentin®) during 10 weeks. For callogenesis and caulogenesis steps, cultures were placed at 24 ± 2°C, under diffuse light (6 μmol m^{-2} s^{-1}, fluorescent tubes Mazdafluor TF″P″/JR and SF/AUR) with a 16 h photoperiod. To allow shoot elongation, organogenic calli were transferred onto BM supplemented with 1 μM GA_3, 70 mg l^{-1} kanamycin and 400 mg l^{-1} Augmentin® for 1 month. Individual shoots were excised and rooted in BM containing 1 μM IBA, 150 mg l^{-1} kanamycin and 400 mg l^{-1} Augmentin® for 1 month. Root elongation was performed on antibiotic-free BM. During shoot elongation and rooting, cultures were placed at 24 ± 2°C, under cool-white fluorescent light (16 h photoperiod, 58 μmol m^{-2} s^{-1}). Rooted plantlets were acclimatized in a greenhouse in pots filled with a mixture of commercial compost and vermiculite (3:1; v/v).

DNA extraction, PCR and southern blot analysis

Genomic DNA was extracted from 1.5 g of leaves of putative transformed plants previously acclimatized and control plants, using the CTAB method (Murray & Thompson, 1980) with the following extraction buffer: 100 mM Tris–HCl pH 7.5, 0.7 mM NaCl, 10 mM Na_2EDTA pH 8, 2% 2-mercaptoethanol and 2% CTAB (Sigma, USA).

Two sets of PCR (polymerase chain reaction) amplifications were carried out to detect the *gus* and *nptII* genes. PCR was performed in 25 μl volumes containing 10 mM Tris–HCl pH 9, 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP, 25 pmol of each oligonucleotide primer, 3.75 units of *Taq* polymerase (Pharmacia Biotech, France) and 100 ng of plant DNA. Amplification of a 469 bp *nptII* gene fragment was obtained with the primers 5′-CAAGATGGATTGCACGCAGGTTC-3′ and 5′-TCCAGATCATCCTGATCGACAAG-3′. The primer set 5′-TAGAAACCCCAACCCGTGAAATC-3′ and 5′-CGACCAAAGCCAGTAAAGTAGAA-3′ allowed the amplification of a 1062 bp *gus* gene fragment. The PCR was carried out in a thermal cycler (Gene Amp 2400, Perkin Elmer, The Netherlands) using an initial 3 min denaturation step at 94°C followed by 35 cycles of 20 s at 94°C, 20 s at 60°C and 1 min 15 s at 72°C with a final extension step of 72°C for 5 min. Amplified products were detected by ultraviolet light

fluorescence (312 nm) after electrophoresis on 1.5% agarose gels stained with ethidium bromide.

The Southern blot analysis was performed with non radioactive digoxigenin labelled probes. DNA from control plants and plants transformed with *Agrobacterium* strain EHA105/GI was digested with *Hin*dIII or *Eco*RI. Twenty micrograms of DNA was run on 0.8% agarose gels and blotted onto nylon membrane (Hybond N^+, Amersham, France) under alkaline conditions according to Clark (1997). Probes used for detecting *nptII* and *gus* transgenes were obtained by amplification of the 469 and 1062 bp fragments as previously described using 1 μg of pMOG410 plasmid as a template in the PCR mixture (Figure 1). The two probes were then digoxigenin-labelled by random priming according to manufacturer's instructions (Boehringer Mannheim, France). Membranes were prehybridized at 65°C for 5 h in hybridization buffer (5× SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, 1% blocking reagent (Boehringer Mannheim, France) supplemented with 200 μg ml^{-1} salmon sperm DNA (Sigma, USA). Hybridization was carried out overnight at 65°C in hybridization buffer containing 20 ng ml^{-1} probe. The membranes were then washed twice for 5 min in 2× SSC, 0.1% SDS at 25°C, twice for 20 min in 0.1× SSC, 0.1% SDS at 68°C and rinsed in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl pH 7.5). Non-specific binding sites were blocked for 1 h in blocking solution (maleic acid buffer, 1% blocking reagent). The membranes were then incubated for 30 min in anti-DIG-AP conjugate (1:5000 dilution in blocking solution) and washed twice for 20 min in maleic acid buffer at room temperature. Chemiluminescent detection with CSPD® was carried out according to manufacturer's instruction (Boehringer Mannheim, France).

Histochemical GUS assay

The GUS assay in putative transgenic plants was carried out with 1 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronide according to Jefferson (1987). The *gus* gene expression was analysed in five randomly chosen leaves per plant. Each leaf was incubated separately to avoid the diffusion of intermediary compounds produced during the GUS reaction (Guivarc'h et al., 1996).

Leaf callus assay

Five randomly selected leaves per transgenic plant were cut into sections 5–7 mm in length and placed on callogenesis medium supplemented with 70 mg l^{-1} kanamycin. After 6 weeks of culture, the percentage of leaves showing callus was recorded.

Plant mRNA isolation and RT-PCR procedure

The extraction of total mRNAs from 30 mg leaf tissue was performed using the RNeasy Plant Mini Kit (Qiagen, France) according to the manufacturer's instructions. Reverse transcription was primed from 0.6 μg oligo$(dT)_{15}$ (Promega, USA) and reactions were carried out in a 25 μl volume containing 50 mM Tris–HCl, 40 mM KCl, 7 mM $MgCl_2$, 10 mM DTT, 0.5 mM of each dNTP, 25 units of RNase inhibitor (Promega, USA) and 200 units M-MLV reverse transcriptase (Promega, USA). PCR amplification of cDNA fragments from the *gus* gene was performed using the conditions previously described (see PCR procedure).

Statistical analysis

The percentages of Kan^r (kanamycin resistant) calli obtained in each set of experimental conditions and the rates of plant regeneration after transformation procedures using three *Agrobacterium* strains (EHA105/GI, C58/GI and AGL1/GI) were recorded. Comparisons were performed by using a chi-square global comparison followed by a *G*-test (Scherrer, 1984).

Results

Influence of the inoculation method and bacterial strain on the recovery of putative transformed callus lines

Our first series of experiments were performed to define the best conditions for the recovery of putative transformed callus lines. With this aim, we compared the influence of different periods of both precultivation (2 or 8 days) and cocultivation (2 or 4 days), and the effects of five strains of *Agrobacterium* (LBA4404/GI, GV2260/GI, AGL1/GI, EHA105/GI and C58/GI). The efficiency of transformation was evaluated as the percentage of Kan^r calli formed after 6 weeks of culture on selective medium. In all cases, calli had the same appearance: they grew rapidly from the cut edges of leaf explants into compact nodular structures (Figure 2a). Two strains (LBA4404/GI and GV2260/GI) were totally inefficient for transformation. The three others (AGL1/GI, EHA105/GI and C58/GI) were

Figure 2. Development of transgenic plants of lavandin. (a) Kanamycin resistant callus obtained after 6 weeks on callogenesis medium supplemented with 70 mg l^{-1} kanamycin and 400 mg l^{-1} Augmentin® , (b) kanamycin resistant callus showing bud (arrow) after 6 weeks on caulogenesis medium containing 70 mg l^{-1} kanamycin and 400 mg l^{-1} Augmentin® , (c) transgenic plant of lavandin after transformation with *Agrobacterium* strain EHA105/GI (left) and non-transformed plants (right). Scale bars: 1 mm.

Table 2. Effect of precultivation, cocultivation and bacterial strains on Kan^r callus percentages obtained after 6 weeks of culture on selective medium

Agrobacterium strains and cocultivation conditions (days)	Percentage* of leaves showing Kan^r calli	
	Precultivation 2 days	Precultivation 8 days
AGL1/GI		
2	0.5	6.0
4	17.0	70.5
EHA105/GI		
2	n.d.	9.0a
4	12.7a	54.0
C58/GI		
2	0.0	15.5
4	5.9	42.5

* Percentages were calculated from 200 to 210 leaf explants for each experimental condition in one replicate (n.d., not determined). The influence of precultivation and cocultivation durations was estimated independently for each bacterial strain. No statistical comparison was shown between the strains in the Table 2. Only values followed by the same letter are not significantly different according to a G-test ($p = 0.05$).

tested for 2 or 4 days of cocultivation with leaf explants which had been precultivated for 2 or 8 days (Table 2). Whichever bacterial strain was used, percentages of Kan^r calli increased with the precultivation and cocultivation periods. Best results were always obtained using an 8-day precultivation and a 4-day cocultivation (G-test, $p = 0.05$). In these optimal conditions, the highest percentage of Kan^r calli (70%), showed statistically different (G-test, $p = 0.05$), was recorded with the AGL1/GI strain, against 40–50% for the C58/GI and EHA105/GI strains.

Regeneration of putative transgenic plants

To induce the regeneration of transgenic plantlets, calli obtained with the three efficient *Agrobacterium* strains (AGL1/GI, EHA105/GI, C58/GI) were cultured on a succession of selective media allowing bud formation (Figure 2b), shoot elongation and root differentiation. The putative transgenic plantlets regenerated were morphologically identical to non-transformed plants (Figure 2c), but their development and growth were slower. As a consequence of this delay, which was probably caused by kanamycin, plantlets was obtained only after 6 months. The frequencies of transformation, calculated as a percentage of leaf explants giving putative transgenic plantlets, were 3.5, 5.5 and 9.0% with bacterial strains AGL1/GI, C58/GI and EHA105/GI respectively. These results were statistically analysed and no significant difference was detected after a chi-square global comparison ($p = 0.05$). Consequently, in this model, no correlation between the number of Kan^r calli obtained and the number of putative transgenic plants regenerated could be found.

Verification of the integration of the gus *and* nptII *transgenes into the lavandin genome and estimation of their copy number*

To confirm the presence of the *nptII* and *gus* genes into the genome of putative transformants, a PCR analysis was carried out on 20 randomly chosen plants (Figure 3). Using the *nptII* primer set, the expected 469 bp fragment was found in all the plants analysed (Figure 3a) whereas with the *gus* primer set, two out of the 20 plants tested gave no PCR amplification product of the expected size (1062 bp; Figure 3b). This result strongly suggests that, out of the 20 plants studied, 18 have integrated both the *nptII* and *gus* genes in their nuclear genome.

To further characterize the putative transformants, 9 plantlets (transformed with EHA105/GI and testing positive for the presence of both *nptII* and *gus* genes by PCR) were randomly taken for Southern blot analysis (Figure 4). In order to verify the integrity of the *gus* gene copies, genomic DNA from transformants P5, P6, P11, P13, P14, P17, P18 and P19 was digested with *Hin*dIII. When hybridized with *gus* probe, an internal fragment of the *gus* gene with the expected size 2700 bp (Figure 4a, lanes 4, 5, 9, 7, 8, 6, 10, 11, respectively) was detected in 8 of the 9 plants, indicating that these plants had integrated at least one intact copy of the transgene. The transformant P4 contained only

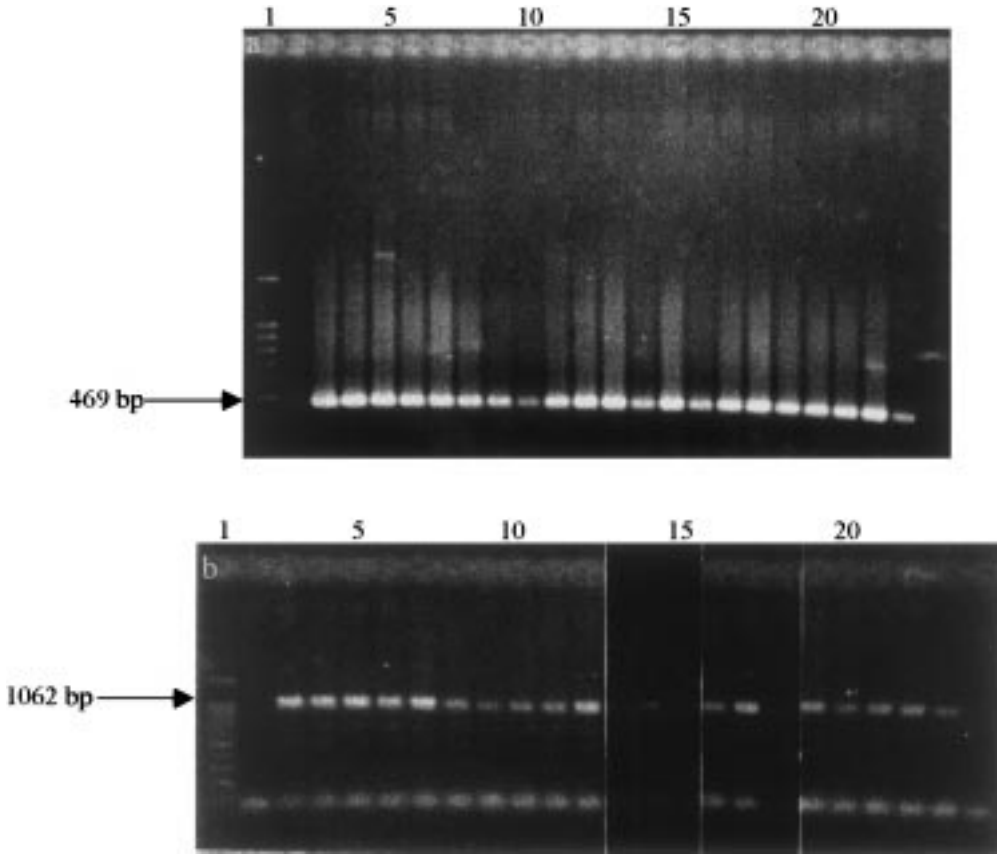

Figure 3. PCR analysis of DNA isolated from leaves of one non-transformed lavandin and 20 transgenic plants. Agarose gel electrophoresis of PCR amplification was performed with primers for the *nptII* gene (a) and with primers for the *gus* gene (b). Lane 1: molecular size marker (100 bp, Promega, USA), Lane 2: non-transformed plant, Lane 3: plasmid pMOG410, Lanes 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23: plants P1, P2, P3, P4, P5, P6, P7, P8, P9, P10, P11, P12, P13, P14, P15, P16, P17, P18, P19, P20 respectively, Lane 24: PCR amplification without DNA.

truncated copies of the *gus* gene in its nuclear genome (Figure 4a, lane 3).

Additional analyses were performed to determine the number of copies of the integrated *gus* and *nptII* genes. Digestions of genomic DNA with *Eco*RI, which had unique restriction site at the 3′-end of the *gus* gene, and subsequent hybridization with either *gus* (Figure 4b) or *nptII* (Figure 4c) probes gave different patterns according to the number of copies integrated. DNA from non-transformed plants used as a negative control showed no hybridization with the two probes (Figure 4a, lane 2 and Figure 4c, lane 32). A single copy of both *gus* and *nptII* genes was carried by plants P5 and P13, while 6 transformants P4, P6, P11, P14, P17 and P18 contained 2–4 copies of each reporter genes. Only plant P19 carried 6–9 copies of *gus* and *nptII* transgenes.

Digestion with *Hin*dIII and subsequent hybridization with the *gus* probe allowed us to determine the number of truncated copies integrated (Figure 4a). Two and four integrated copies of *gus* genes could be detected in the transformants P11 and P14 (Figure 4a, lanes 9 and 8 respectively), whereas only 1 or 2 highly intense bands, corresponding probably to two transgene copies of the same size, were observed after a digestion with *Eco*RI (Figure 4b, lanes 19 and 18). In the same way, plant P17 had integrated four copies of *gus* gene (Figure 4a, lane 5), although only one band was obtained after a digestion with *Eco*RI (Figure 4b, lane 16). Southern blot analysis involved the use of an accurately determined concentration of DNA. Thus, the presence of only one band for transformant P17 when its genomic DNA was digested by *Eco*RI and hybridized with the *gus* probe was certainly due to the

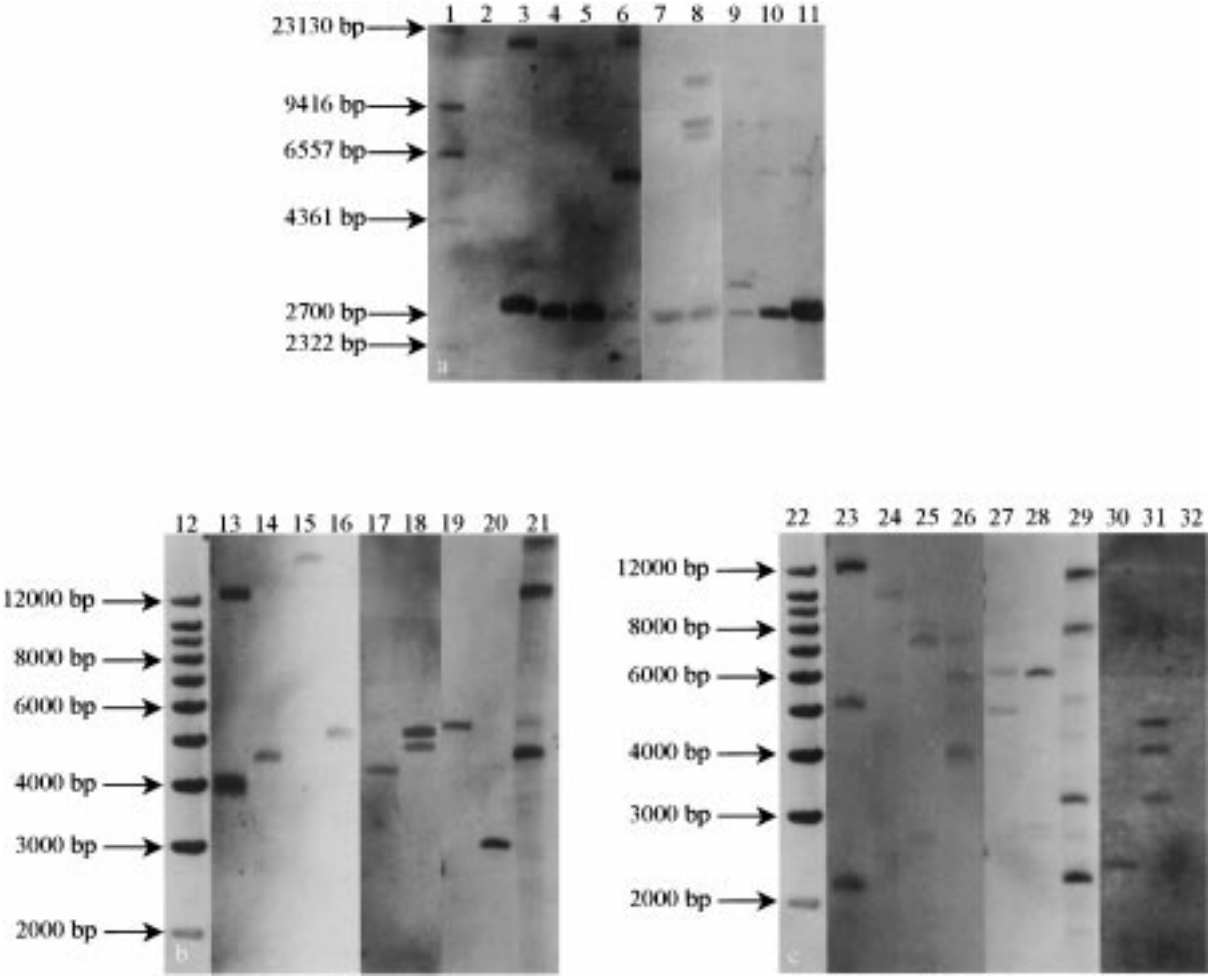

Figure 4. Southern blot analysis of DNA isolated from leaves of one untransformed control and nine plants transformed with *Agrobacterium tumefaciens* EHA105/GI. Total genomic DNA was digested with *Hind*III (a) and *Eco*RI (b,c). Digested DNAs were hybridized with digoxigenin labelled probes detecting either the *gus* gene (a, b) or the *nptII* gene (c). Lane 1: molecular size marker (Boehringer Mannheim, France), Lanes 12 and 22: molecular size marker (12 kbp, Stratagene, USA) Lanes 2 and 32: non-transformed lavandin, Lanes 3, 13 and 23: plant P4, Lanes 4, 14 and 24: plant P5, Lanes 5, 15 and 25: plant P6, Lanes 6, 16 and 26: plant P17, Lanes 7, 17 and 30: plant P13, Lanes 8, 18 and 31: plant P14, Lanes 9, 19 and 27: plant P11, Lanes 10, 20 and 28: plant P18, Lanes 11, 21 and 29: plant 19.

use of a low quantity of DNA. The overall results of Southern blot analyses are summarized in Table 3.

Analysis of gus and nptII genes expression

Histoenzymatic GUS assay and leaf callus assays were carried out to investigate expression of the *gus* and *nptII* genes, respectively, in the nine transgenic plants analysed by Southern blot. In these experiments, five randomly chosen leaves per transformant were used. For each of the nine plants studied, every leaf tested gave callus when cultured in the presence of 70 mg l^{-1} kanamycin (Table 3). Callus formation was not observed on leaves from non-transformed plants. The results of the GUS assays were slightly more complex (Table 3): three plants (P4, P13 and P18) exhibited a β-glucuronidase activity in all the leaves tested (Figure 5), three plants (P11, P14 and P17) were GUS^- (*i.e.* no β-glucuronidase activity was detected in the GUS assay) and the three remaining plants (P5, P6 and P19) had both GUS^- and GUS^+ leaves. No intrinsic GUS activity was detected in leaves of non-transformed plants.

As the lack of GUS activity could have various explanations, RT-PCR was carried out to check the presence of *gus* gene transcripts in the 3 GUS^- plants (P11, P14 and P17), in two $GUS^{\pm}$ plants (P6 and P19) and in two GUS^+ plants as a control (P4 and P13).

Table 3. Analysis in nine independent transgenic plants of lavandin for the integrity of *gus* gene, the number of integrated copies of *gus* and *nptII* genes and the expression of these transgenes

Plant code	Full copy (+) or truncated copy (−) of *gus* gene	Copy number of *gus* gene	Copies number of *nptII* gene	GUS assay	Leaf callus assay
Control plant	n.d.	0	0	−	−
P13	+	1	1	+	+
P5	+	1	1	+/−	+
P6	+	1	2	+ / −	+
P18	+	2	2/3	+	+
P4	−	2/3	3	+	+
P17	+	4	4	−	+
P11	+	2	2	−	+
P14	+	4	4	−	+
P19	+	6/9	6/9	+/−	+

Histoenzymatic GUS analysis and leaf callus assay were performed with five randomly chosen leaves from each transgenic plant of lavandin.
+: All leaves tested from one plant were kan^r or GUS positive, +/−: Plants with some GUS positive leaves, −: Plants with all GUS negative leaves. n.d. Non determined.

The intron present in the *gus* construct is 189 bp in length (Vancanneyt et al., 1990), so that an amplification product of 873 bp is expected in spliced cDNA. RT-PCR analysis (Figure 6) of the non-transformed plants never gave any amplification product, whereas the expected 873 bp fragment was detected in the two GUS^+ plants (Fig. 6, lanes 5 and 6). Despite the fact that according to the Southern blot results, the GUS^+ transformant P4 had only integrated truncated copies of the *gus* gene, *gus* gene expression appeared to be normal in this plant. In plants with both GUS^+ and GUS^- leaves, the 873 bp fragment amplified from the transcript product of the *gus* gene was detected by RT-PCR (Figure 6, lanes 8 and 10). Finally, in the three GUS^- plants which had integrated at least a full copy of the *gus* gene, the presence of the 873 bp amplified fragment was detected in plants P14 and P17, but not in plant P11 (Figure 6, lanes 7, 9 and 11, respectively). Consequently, the lack of GUS activity was explained by the absence of production of the *gus* gene transcript only in plant P11.

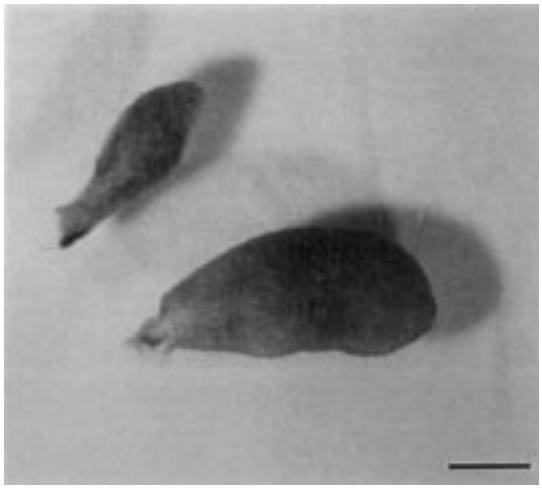

Figure 5. Histoenzymatic detection of the *gus* gene expression in leaves of transgenic plant of lavandin. Scale bar: 1 cm.

Chimerism evaluation of transgenic plants

Histoenzymatic GUS assay and leaf callus assay were used again to evaluate the chimerism of transgenic plants obtained with our transformation protocol. These experiments were carried out using batches of five randomly chosen leaves taken from 17 independent transformants, for which PCR analysis had previously shown the presence of both *nptII* (Figure 3a) and *gus* (Figure 3b) genes in the nuclear genome. All transgenic plants tested regenerated calli from all explants cultured on selective medium whereas no callus proliferation was observed from leaves of non-transformed plants (Table 4). The high selection pressure used during transformants rooting avoided but did not eliminate the formation of plants chimeric for *nptII* transgene. The expression of the *gus* gene

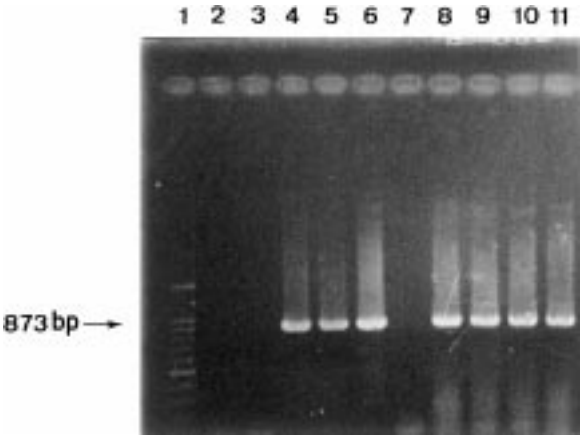

Figure 6. Agarose gel electrophoresis of RT-PCR fragments amplified from the transcript product of the *gus* gene. Lane 1: molecular size marker (100 bp, Promega, USA), Lane 2: PCR amplification without DNA, Lane 3: mRNA from non-transformed lavandin, Lane 4: PCR amplification of pBI 121, Lane 5: mRNA from plant P4, Lane 6: mRNA from plant P13, Lane 7: mRNA from plant P11, Lane 8: mRNA from plant P19, Lane 9: mRNA from plant P14, Lane 10: mRNA from plant P6 and Lane 11: mRNA from plant P17.

Table 4. Analysis of the *gus* and *nptII* gene expression in leaves from 17 transgenic plants of lavandin

leaves	No. of plants with all positive leaves	No. of plants with some positive leaves	No. of plants with all negative
GUS assay	7/17	6/17	4/17
Leaf callus assay	17/17	0/17	0/17

Histoenzymatic GUS analysis and leaf callus assay were performed with five randomly chosen leaves from each transgenic plants of lavandin.

varied between transgenic plants: Seven transformants were GUS^+, four were GUS^- and six showed both GUS^+ and GUS^- leaves (Table 4). In this case, no selection pressure could be used to prevent the regeneration of plants chimeric for the *gus* gene. Unfortunately, because of the sterility of lavandin, it is impossible to segregate plants regenerated, as done in other species.

Discussion

An efficient *Agrobacterium*-mediated transformation procedure has been established for the first time in lavandin cultivar 'Grosso 2'. The optimization of the T-DNA delivery efficiency has shown that the production of transgenic plants is highly influenced by several parameters such as the durations of precultivation and cocultivation and the *A. tumefaciens* strains used. Results showed that an increase in the precultivation period was correlated with an enhanced production of putative transformed callus lines. A similar observation was reported by VanWordragen & Dons (1992) in many species and could be explained by a reduction in wounding stress. In the same way, a significant increase in the frequency of Kan^r callus regeneration was obtained after lengthening the cocultivation period. This benefical effect was observed and reported both in woody (DeBondt et al., 1994) and herbaceous (Cheng et al., 1997; Nishibayashi et al., 1996; Takasaki et al., 1997) species. Furthermore, Sangwan et al., (1992) in *Arabidopsis thaliana*, Guivarc'h et al., (1993) in *Daucus carota* and DeKathen & Jacobsen (1995) in *Pisum sativum* have shown that transformation competent cells are most frequently dedifferentiated and in division. The increased cell division activity in leaf explants during longer precultivation and cocultivation periods might enhance the proportion of transformation competent cells.

To our knowledge, the susceptibility of lavandin to *A. tumefaciens* infection has never been investigated (DeCleene & DeLey, 1976; VanWordragen & Dons, 1992). Consequently five distinct *Agrobacterium* strains (LBA4404/GI, C58/GI, GV2260/GI, EHA105/GI and AGL1/GI) were used to study the T-DNA delivery and stable genetic transformation of lavandin. These strains included the principal opine types and together their host ranges cover almost all species for which gene transfer has already been reported (VanWordragen & Dons, 1992; Hood et al., 1993). *Agrobacterium*-mediated transformation of lavandin was dependent on the bacterial strain used. The reason for this could be the origin of the bacteria: strains containing a disarmed virulence plasmid of either nopaline (C58/GI) or L,L succinamopine (EHA105/GI or AGL1/GI) type were efficient for lavandin transformation whereas *Agrobacterium* strains carrying an octopine pTi plasmid (LBA4404/GI and GV2260/GI) were avirulent. Strain EHA105/GI gave the highest transformation rate (9%, *i.e.* the number of transgenic plantlets on the total number of initial explants). The disarmed strains of type EHA, derived from the supervirulent strain A281 containing the virulence plasmid pTiBO542 (Hood et al., 1993) have been found to be more virulent than other strains in many species,

e.g., *Malus* x *domestica* (DeBondt et al., 1994), *M.* x *piperita* (Diemer et al., 1998; Nui et al., 1998), *Lycopersicon esculentum* (Hood et al., 1993), *Brassica rapa* (Takasaki et al., 1997). This supervirulence was correlated with an enhanced level of the expression of *virG* (Jin et al., 1987), the gene products of which were necessary for the activation of inducible virulence genes (Sheng & Citovsky, 1996).

Stable integration of the *gus* and *nptII* transgenes into the lavandin genome was confirmed both by PCR and Southern blot analysis. Among the nine transgenic plants studied, Southern blot analysis showed different hybridization patterns, indicating that T-DNAs were randomly integrated into the lavandin genome. At least one full copy of the *gus* gene was detected in the genomic DNA of eight out of the 9 transformants analysed. Moreover, two transgenic plants had integrated one copy of each transgene whereas the six others carried 2–9 copies of the *nptII* and *gus* genes.

For theses nine transgenic plants, transgene expression was studied using a leaf callus assay and histoenzymatic GUS analysis. Results showed that the *nptII* gene was expressed in all transformants at a sufficiently high level to allow a cellular proliferation on selective medium. In contrast, the level of *gus* gene expression varied between the transgenic plants studied. GUS activity was never detected in leaves of three transformants (P11, P14 and P17), which nevertheless contained a full copy of the *gus* gene in their nuclear genome. The subsequent RT-PCR analysis of the plant P11 showed that the corresponding transcript had not accumulated in its leaves. Such lack of transcription could have many origins (Maessen, 1997; Stam et al., 1997): hypermethylation, transgene integration in a non-coding region (heterochromatin) of the genome, suppression of expression by nearby regulatory sequences of endogenous genes or inactivation due to the presence of multiple copies. In plants P14 and P17, RT-PCR analysis showed the presence of the transcript product of the *gus* gene, although no GUS activity was detected in their tissues. Since RT-PCR primed with oligo(dT) did not provide any information about transcript size, it is possible that mRNAs which are truncated at the 5′-end could be synthetized. If this was true, no active protein would accumulate in leaves of transgenic plants P14 and P17. Alternatively, a point mutation in the *gus* gene sequence could result in the production of an aberrant mRNA giving a non-functional protein. Finally, the lack of GUS activity could also be explained by post-transcriptional gene silencing. Four copies of the *gus* gene were integrated in the genomic DNA of plants P14 and P17. These transgenes could produce so much mRNA that the level exceeded a critical threshold thereby triggering a mechanism of mRNA degradation (Stam et al., 1997).

The six other transgenic plants tested showed *gus* gene expression in their leaves. However, in plants P5, P6 and P19, GUS activity varied between leaves. A similar observation was made in transgenic wheat (Cheng et al., 1997). There are several possible explanations for this variable GUS activity. Problems of substrate penetration and oxidation after an overnight incubation in X-Glu were observed in lavandin, as reported in leaves of sweet orange (Cervera et al., 1998). Such artefacts in histochemical GUS assays, known as impossible to avoid, have been well documented by Guivarc'h et al., (1996). Finally, these transgenic lavandin plants could be chimeric, with only some regions containing the *gus* reporter gene.

In conclusion, we have developed, for the first time in lavandin, a method allowing the rapid production of transgenic plants using *Agrobacterium tumefaciens*. The transformation rates, based on the percentages of transgenic plants regenerated, ranged from 3.5 to 9.0% with three bacterial strains (AGL1/GI, C58/GI and EHA105/GI) and the transformed plants appeared to be morphologically identical to non-transformed plants. The stable integration of the *nptII* and *gus* genes was confirmed both by PCR and Southern blot analysis. Moreover, the study of transgene expression by the histoenzymatic GUS detection and leaf callus assay showed that at least seven transformants out of 17 expressed both the *nptII* and *gus* genes at a high level.

This efficient transformation procedure established in lavandin could be used to produce transgenic plants with altered expression of genes encoding for enzymes of the monoterpene pathway. This type of manipulation could allow qualitative or quantitative modification of the composition of lavandin essential oils.

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References

Bird CR, Ray JA, Fletcher JD, Boniwell JM, Bird AS, Teulieres C, Blain I, Bramley PM and Schuch W (1991) Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes. *Bio/Technology* **9**: 635–639.

Boelens MH (1995) Chemical and sensory evaluation of *Lavandula* oils. *Perfumer and Flavorist* **20**: 23–51.

Bohlmann J, Steele CL and Croteau R (1997) Monoterpene synthases from grand fir (*Abies grandis*) *J Biol Chem* **272**: 21784–21792.

Cervera M, Juarez J, Navarro A, Pina JA, Duran-Vila N, Navarro L and Pena L (1998) Genetic transformation and regeneration of mature tissues of woody fruit plants bypassing the juvenile stage. *Transgenic Res.* **7**: 51–59.

Cheng M, Fry JE, Pang S, Zhou H, Hironaka CM, Duncan DR, Conner TW and Wan Y (1997) Genetic transformation of wheat mediated by *Agrobacterium tumefaciens*. *Plant Physiol* **115**: 971–980.

Clark MS (1997) Southern blotting. In: Clark MS (ed.), *Plant molecular biology: a laboratory manual* (pp.15–25) Springer-Verlag, Berlin.

Colby SM, Alonso WR, Katahira EJ, McGarvey DJ and Croteau R (1993) 4*S*-limonene synthase from oil glands of spearmint (*Mentha spicata*). *J Biol Chem* **268**: 23016–23024.

Daudonnet S, Karst F and Tourte Y (1997) Expression of the farnesyldiphosphate synthase gene of *Saccharomyces cerevisiae* in tobacco. *Mol Breed* **3**: 137–145.

Deblaere R, Bytebier B, Beboeck F, Schell J, VanMontagu M and Leemans J (1985) Efficient octopine Ti plasmid-derived vectors for *Agrobacterium*-mediated gene tranfer to plants. *Nucl. Acids Res* **13**: 4777–4788.

DeBondt A, Eggermont K, Druart P, DeVil M, Goderis I, Vanderleyden J and Broekaet F (1994) *Agrobacterium*-mediated transformation of apple (*Malus* x *domestica* Borkh.): an assessment of factors affecting gene transfer efficiency during early transformation steps. *Plant Cell Rep* **13**: 587–593.

DeCleene M and DeLey J (1976) The host range of crown gall. *Bot. Rev.* **42**: 389–466.

DeKathen A and Jacobsen H-J (1995) Cell competence for *Agrobacterium*-mediated DNA transfer in *Pisum sativum* L. *Transgenic Res* **4**: 184–191.

Diemer F, Jullien F, Faure O, Moja S, Colson M, Matthys-Rochon E and Caissard J-C: 1998, High efficiency transformation of peppermint (*Mentha* x *piperita* L.) with *Agrobacterium tumefaciens*. *Plant Sci*. **136**: 101–108.

Dronne S, Jullien F, Caissard J-C and Faure O (1999) A simple and efficient method for *in vitro* shoot regeneration from leaves of lavandin (*Lavandula* x *intermedia* Emeric ex Loiseleur). *Plant Cell Rep*. **18**: 429–433.

Dudareva N, Cseke L, Blanc VM and Pichersky E (1996) Evolution of floral scent in *Clarkia:* novel patterns of *S*-linalool synthase gene expression in the *C. breweri* flower. *Plant Cell* **8**: 1137–1148.

Guivarc'h A, Caissard JC, Azmi A, Elmayan T, Chriqui D and Tepfer M (1996) *In situ* detection of expression of the *gus* reporter gene in transgenic plants: ten years of blue genes. *Transgenic Res*. **5**, 281–288.

Guivarc'h A, Caissard JC, Brown S, Marie O, Dewitte W, Van Hockelen H and Chriqui D (1993) Localization of target cells and improvement of *Agrobacterium*-mediated transformation efficiency by direct acetosyringone pretreatment of carrot root discs. *Protoplasma* **174**: 10–18.

Hoekema A, Hirsch PR, Hooykaas PJJ and Schilperoort A (1983) A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* **303**: 179–180.

Hood EE, Gelvin SB, Melchers LS and Hoekema A (1993) New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Res* **2**: 208–218.

Jefferson RA (1987) Assaying chimearic genes in plants: the GUS gene fusion system. *Plant Mol Biol* **204**: 387–405.

Jin S, Komari T, Gordon MP and Nester EW (1987) Genes responsible for the supervirulence phenotype of *Agrobacterium tumefaciens* A281. *J Bacteriol* **169**: 4417–4425.

Koncz C and Schell J (1986) The promoter of T_L-DNA gene *5* controls the tissue-specific expression of chimearic genes carried by a novel type of *Agrobacterium* binary vector. *Mol Gen Genet* **204**: 383–396.

Lawrence BM (1994) Progress in essentiel oils. *Perfumer and Flavorist* **19**: 83–95.

Lazo GR, Stein PA and Ludwig RA (1991) A DNA transformation-competent *Arabidopsis* genomic library in *Agrobacterium*. *Bio/Technology* **9**: 963–967.

Maessen GDF (1997) Genomic stability and stability of expression in genetically modified plants. *Acta Bot Neerl* **46**: 3–24.

McCaskill D and Croteau R (1997) Prospects for bioengineering of isoprenoid biosynthesis. In: Scheper T (ed.), Advances in biochemical engineering biotechnology, Vol 55 (pp.101–146) Springer-Verlag, Berlin.

McGarvey DJ and Croteau R (1995) Terpenoid metabolism. *Plant Cell* **7**: 1015–1026.

Morel G and Wetmore RH (1951) Tissue culture of monocotyledons. *Am J Bot* **38**: 138–140.

Murashige T and Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* **15**: 473–497.

Murray MG and Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* **8**: 4321–4325.

Nishibayashi S, Kaneko H and Hayakawa T (1996) Transformation of cucumber (*Cucumis sativus* L.) plants using *Agrobacterium tumefaciens* and regeneration from hypocotyl explants. *Plant Cell Rep* **15**: 809–814.

Niu X, Lin K, Hasegawa PM, Bressan RA and Weller SC (1998) Transgenic peppermint (*Mentha* x *piperita* L.) plants obtained by cocultivation with *Agrobacterium tumefaciens*. *Plant Cell Rep* **17**: 165–171.

Sangwan RS, Bourgeois Y, Brown S, Vasseur G and Sangwan-Norreel B (1992) Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana Planta* **188**: 439–456.

Schaller H, Grausem B, Benveniste P, Chye ML, Tan CT, Song YH and Chua NH (1995) Expression of the *Hevea brasiliensis* (H.B.K.) Müll. Arg. 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 in tobacco results in sterol overproduction. *Plant Physiol* **109**: 761–770.

Scherrer B (1984) *Biostatistique* Montréal: Gaëtan Morin.

Segura J and Calvo MC (1991) *Lavandula* spp. (Lavender): *in vitro* culture, regeneration of plants, and the formation of essential oils and pigments. In: Bajaj YPS (ed.), Biotechnology in Agriculture and Forestry Vol. 15 (pp.283–310) Springer-Verlag, Berlin.

Sheng J and Citovsky V (1996) *Agrobacterium*-plant cell DNA transport: Have virulence proteins, will travel. *Plant Cell* **8**: 1699–1710.

Stam M, Mol JNM and Kooter JM (1997) The silence of genes in transgenic plants. *Ann Bot* **79**: 3–12.

Takasaki T, Hatakeyama K, Ojima K, Watanabe M, Toriyama K and Hinata K (1997) Factors influencing *Agrobacterium*-mediated transformation of *Brassica rapa* L. *Breeding Sci.* **47**: 127–134.

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L and Rocha-Sosa M (1990) Construction of an intron-containing marker gene: splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. *Mol Gen Genet* **220**: 245–250.

VanWordragen MF and Dons HJM (1992) *Agrobacterium tumefaciens*-mediated transformation of recalcitrant crops. *Plant Mol Biol Rep* **10**: 12–36.

Wise ML, Savage TJ, Katahira E and Croteau R (1998) Monoterpene synthases from common sage (*Salvia officinalis*) *J Biol Chem* **273**: 14891–14899.