Theor Appl Genet (1999) 99:676–682

S. Krasnyanski · R.A. May · A. Loskutov
T.M. Ball · K.C. Sink

Transformation of the limonene synthase gene into peppermint (*Mentha piperita* L.) and preliminary studies on the essential oil profiles of single transgenic plants

Received: 22 November 1998 / Accepted: 4 Januar 1999

Abstract *Agrobacterium*-mediated and direct gene transfer into protoplasts using PEG were both successfully used to produce stable, transformed peppermint plants (*Mentha×piperita* L. cultivar Black Mitcham) with the limonene synthase gene. Stem internode explants found to possess a high level of organogenesis through adventitious shoot formation were subjected to *Agrobacterium tumefaciens* disarmed strain GV3101 (pMP90). Following the development of an efficient protoplast-to-plant cycle from stem-isolated protoplasts, they were used in direct gene transformations. In both cases the binary vector pGA643 carrying the *npt*II/GUS genes, both driven by the CaMV35S promoter, was used in preliminary plant-transformation studies. Later, GUS was replaced with the limonene synthase gene. Kanamycin was used as a selective agent in all transformation experiments to obtain both transformed protoplast-derived calli as well as putative transgenic shoots regenerated from internode explants. Both types of transformation resulted in transgenic plants which were detected using PCR and confirmed by Southern-blot hybridizations. Southern analysis revealed that the method of *Agrobacterium*-mediated transformation is superior to the direct DNA uptake into protoplasts with regard to the stability of the insert during the transformation event. Single transgenic plants were grown to 10% flowering in a greenhouse and the plants derived both by the *Agrobacterium* and the protoplast-derived methods were generally observed to have essential oil profiles characterized by a high-menthone, low-menthol, high-menthofuran and –pulegone content in comparison to a typical mid-west peppermint. Limonene varied only slightly, around 1.2%, in transgenic plants produced by both methods.

Key words *Agrobacterium*-mediated transformation · Direct gene transfer · Essential oils · Limonene synthase · Peppermint

Communicated by Y. Gleba

S. Krasnyanski
NRES, University of Illinois, 1201 W. Gregory, Urbana, IL 61801, USA

R.A. May · A. Loskutov · T.M. Ball · K.C. Sink (✉)
Department of Horticulture, Michigan State University, E. Lansing, MI 48824, USA
e-mail: sink@pilot.msu.edu
Fax: +517 353 0890

Introduction

Mentha×piperita cv Black Mitcham is the most widely cultivated peppermint in the United States for oil production. The essential oils that give peppermint its characteristic aroma and flavor are produced in glandular trichomes and are primarily composed of monoterpenes. Mint oil is used worldwide mostly in the confectionary and pharmaceutical industries. Because of the constant market demand for this oil, it is of great interest to determine the influence of introduced genes on this pathway on metabolism as well as on oil production. Such efforts via conventional breeding methods have been unsuccessful in developing a higher oil-yielding peppermint that has the oil qualities of Black Mitcham. Mint cultivar development has been traditionally achieved by mutation breeding because peppermints are highly sterile and are propagated vegetatively. However, new cultivar production and evaluation can take 10–15 years using this method and the mutagen may cause undesirable changes such as altering the oil profile and increased disease susceptibility. Genetic engineering has the potential for developing mint cultivars with enhanced oil yield in a shorter period of time without altering other favorable production characteristics.

Limonene is the precursor to all essential oil components in both peppermint and spearmint. It is one of the simplest of the cyclic monoterpenes and is very widely distributed in the plant kingdom being found in the essential oil of members of the Laminaceae (mints), Rutaceae (citrus), Pinaceae (conifers), as well as in *Angelica archangelica* among the Apiaceae (Umbelliferae) family (Guenther 1972). Limonene synthase, a cyclase enzyme

of the mint oil biosynthesis pathway, is considered rate-limiting and determines the overall rate of oil biosynthesis and the yield of essential oils (Gershenzon and Croteau 1991). It catalyzes the reactions by which geranyl pyrophosphate (GPP) is cyclized to limonene (Kjonas and Croteau 1983). This enzyme is also developmentally regulated and is highest in extracts of young leaf tissue where biosynthetic capacity progressively decreases with leaf expansion and maturation (Massimo et al. 1989; Croteau 1991). Transforming a mint limonene synthase gene under the control of a constitutive promoter back into mint may increase the overall conversion of GPP, and maintain expression of the enzyme in mature tissues.

Agrobacterium tumefaciens wild-type strains and a disarmed strain containing the *ipt* gene have been used to transform shoot cultures of *Mentha citrata* and *M.*×piperita that were subsequently studied for monoterpene accumulation and yield (Spencer et al. 1990, 1993). Transient GUS expression studies following co-cultivation of apical leaves of peppermint, but without the production of transgenic plants, were carried out by Caissard et al. (1996). Stable transformation of peppermint has been demonstrated using the reporter genes GUS/MOG and NPTII with a low efficiency, 1 or 10% (Diemer et al. 1998; Niu et al. 1998) respectively, using *Agrobacterium*. In the former study, both the microprojectile and *Agrobacterium* methods resulted in transient GUS expression, but only *Agrobacterium* transformation yielded stable transgenic plants.

In the present study, we have established two reproducible protocols for the transformation of *Mentha piperita* cv Black Mitcham with the limonene synthase gene: *Agrobacterium* transformation and direct gene transfer into protoplasts using PEG. We report that both methods resulted in the production of stable transgenic mint plants carrying the introduced limonene synthase gene.

Materials and methods

Plant material

Rhizomes (about 8 cm) of *Mentha*×*piperita* cv Black Mitcham (Summit Plant Laboratories, Inc., Fort Collins, CO) were potted, three each, in a 7.5-l plastic nursery pot containing Bacto Mix potting soil. The stock plants were maintained in the greenhouse at a 18°C day/16°C night regime, fertilized weekly with 200 ppm of N from Peters 20-20-20, and given supplemental lighting provided by high-pressure sodium lamps to provide a 16-h photoperiod. The stock plants were trimmed monthly and re-potted every 3 months to promote vigorous growth.

In vitro stock plants

Shoots of greenhouse-grown plants were harvested, stripped of leaves and washed for 5 min in 0.1% Sigmaclean soap. The shoots were rinsed with water and then placed in 70% ethanol for 1 min. The ethanol was subsequently removed and replaced with 10% Clorox plus 0.1% Tween 80. The shoots were surface-sterilized for 20 min followed by three rinses in sterile distilled water. Nodal explants, 2-cm long, were placed individually into 15×2.0 cm culture tubes each containing 15 ml of EM medium. EM medium was used for the maintenance and rooting of Black Mitcham shoots and contained MS salts (Murashige and Skoog 1962) with Gamborg et al. (1968) vitamins, 2% sucrose and 0.35% Phytagel (Sigma), pH 5.7. The cultures were incubated at 26°C and 25 $\mu mol \cdot m^{-1} \cdot s^{-1}$ of light provided by Philips F96T12/CW cool white bulbs on a 16-h photoperiod. After 3 weeks, elongated axillary shoots (3–4 cm) were removed from the original nodal explants and inserted, nine each, into a GA-7 Magenta vessel containing 60 ml of EM medium. The shoots were re-propagated by axillary and terminal shoot cuttings every 2 weeks onto fresh EM medium.

Vector construct

The 2.2-kb 4S-limonene synthase (LS) cDNA from spearmint (obtained from R. Croteau, Washington State University, Pullman, WA) was received in pBluescript II SK (+), and designated pLC5.2 (Colby et al. 1993). The binary vector pGA643 containing the *npt*II gene and a polycloning site flanked by the CaMV35S promoter as well as the transcript termination regions of genes 5 and 7 from pTiA6 was obtained from G. An, Washington State University, Pullman, WA (An et al. 1988). PLC5.2 was digested with *Xba*I and *Pvu*II to produce a 2.4-kb fragment containing the LS gene. PGA643 was digested with *Hpa*I and *Xba*I in the polylinker region. The *Xba*I–*Pvu*II fragment from pLC5.2 was ligated to the *Xba*I–*Hpa*I sites in between the 35S promoter and the terminator region in pGA643 to produce the 14-kb binary vector, pGALS. Using the freeze-thaw method (Chen et al. 1994) the binary vector was transformed into *Agrobacterium* strain GV3101 (pMP90) obtained from Dr. C. Koncz (Max-Planck Inst., Germany).

Agrobacterium transformation

The GV3101 (pMP90):pGALS was prepared for explant inoculation by first obtaining single colonies on AB-sucrose minimal medium (Chilton et al. 1974) plus antibiotics, followed by growth of a single colony overnight in 5 ml of YEP medium plus antibiotics. Two milliliters of overnight culture were placed in 25 ml of AB-sucrose liquid medium plus antibiotics and grown until an optical density (*D*) at 600 nm of 0.8–1.0 was reached. Ten milliliters of the culture were then centrifuged (10 min, 3000 g) and re-suspended in liquid AB without antibiotics. For growth and maintenance of GV3101 (pMP90):pGALS the antibiotics gentamicin, rifampicin and kanamycin were used at concentrations of 50, 10 and 50 $\mu g\ ml^{-1}$, respectively.

Internodes from in vitro grown shoots of Black Mitcham peppermint were placed in 25 ml of AB with re-suspended *Agrobacterium* at a density of 1×10^6 cfu/ml, immersed and scored every 1–2 mm with a scalpel blade, and then cut into 5–7-mm segments. The explants were inoculated with *Agrobacterium* for 20–30 min at 28°C in darkness. Following inoculation, the explants were blotted dry on sterile Whatmann #1 filter paper and placed into 100×15 mm Petri dishes each containing 30 ml of D4 medium [MS salts+Gamborg vitamins, sucrose 20 g l^{-1}, coconut water 25.0%, agar 0.7%, benzyladenine (BA) 2 mg l^{-1} and thidiazuron (TDZ) 3 mg l^{-1}, pH 5.7] (Krasnyasnki et al. 1998). The explants were then co-cultivated for 48 h at 25°C in darkness. Following co-cultivation, the explants were washed with liquid MS containing 500 mg l^{-1} of timentin, blotted dry on sterile filter paper and placed in 100×15 mm dishes containing D4 medium with 300 mg l^{-1} of timentin and 20 mg l^{-1} of kanamycin. All explants were incubated at 25°C in darkness.

Direct gene transfer

Protoplasts were isolated according to Krasnyanski et al. (1998) from the 2–3 youngest internode stem segments of Black Mitcham plants maintained in vitro as already described. Washed and puri-

fied protoplasts were used in the direct gene-transfer technique based on the method of Negrutiu et al. (1987) with some modifications. Protoplasts were washed once in CAM solution (0.2 M $CaCl_2 \times 2H_2O$, 0.1% MES) and re-suspended in CAW at 10^6/ml or 4×10^5/sample (0.4 ml). Plasmid DNA (1 ng μl^{-1}), 40 µl total, was added to the protoplasts and mixed gently by shaking. Then, 400 µl of PEG [40% w/v PEG 6000 in 0.4 M sorbitol and 0.1 M Ca $(NO_3)_2$, pH 8.0, filter sterilized] was slowly added, and mixed well by shaking for several seconds. After incubation for 15 min, KSm washing solution was added (10 ml) and protoplasts were purified by centrifugation (80 g, 2 min).

Purified protoplasts were imbedded (5×10^4 prot/ml) in 0.5% melted agarose and cultured according to Krasnyanski et al. (1998). After 4 weeks, protoplast-derived colonies were released from agarose and plated onto solid D4–15 regeneration medium (MS salts+B5 vitamins, sucrose 20 g l^{-1}, coconut water 25.0%, agar 0.7%, BA 2 mg l^{-1} and TDZ 3 mg l^{-1}, pH 7.0) containing 15 mg l^{-1} of kanamycin. After 5 weeks of culture in the dark, surviving calli with shoot buds were transferred onto 5T-15 shoot-development medium (MS salts+B5 vitamins, sucrose 20 g l^{-1}, coconut water 25%, agar 0.7%, BA 1 mg/l, TDZ 2 mg l^{-1}, kanamycin 15 mg l^{-1}, pH 7.0) and incubated under the light. Calli with emerging regenerated shoots were transferred to EM medium without kanamycin for shoot elongation and rooting.

DNA extraction and PCR

Genomic DNA was isolated from young leaves of putative transformed plants grown and maintained in vitro using the standard miniprep protocol of CTAB chloroform/isoamyl extraction according to Doyle and Doyle (1990). PCR was carried out in a Perkin Elmer System 9600. Amplification parameters were 94°C for 5 min followed by 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. A 72°C-incubation for 15 min as a final step was included. The 25-µl amplification mixture contained 100–200 ng of plant genomic DNA. The primers used for amplification of 0.72-kb fragment of the *npt*II gene were 5′-GAG-GCT-ATT-CGG-CTA-TGA-CTG-3′ and 5′-TAG-AAG-GCG-ATG-CGC-TGC-GA-3′. For amplification of the 0.56-kb total fragment of the CaMV 35S promoter (0.34 kb) and the limonene synthase gene (0.22 kb) primers 5′-TGC-CCA-GCT-ATC-TGT-CAC-TTC-3′ and 5′-TGC-CCA-GCT-ATC-TGT-CAC-TTC-3′, respectively, were employed.

Southern-blot analysis

Genomic DNA for Southern hybridization was isolated according to the CTAB extraction procedure (Doyle and Doyle 1990) except that leaf tissues were processed by the ”pasta method” (Hokansen et al. 1977). Extracted DNA was quantified and 8–ng were digested overnight with *Eco*RV and electrophoresed in a 0.9% agarose gel in TAE buffer. DNA was transferred to a nylon membrane (Hybond N+, Amersham) using the capillary blotting technique. A 1.046-kb fragment of pGALS was generated by PCR for use as the limonene synthase probe in Southern hybridization. This fragment spans the junction of the 35S promoter and the coding region of LS gene in pGALS. The probe contained 156 bp of the 35S promoter and the remainder was from the LS coding region. This probe was labeled with ^{32}P and used for hybridization. Blotting, labeling, hybridization and washing were carried out according to Sambrook et al. (1989). The molecular sizes of the hybridized bands were determined in accordance with the inversely proportional relationship of its electrophoretic mobility to the $\log_{10}$ of the number of base pairs (Helling et al. 1974). Lambda-DNA with the restriction endonucleases *Eco*RI and *Hin*dIII was used as the marker to obtain the curves of this relationship.

Essential-oil analysis

Between 50 and 100 g of fresh leaf tissue was collected from greenhouse-grown plants that were approximately 10% in flowering. The leaves were placed into a 500-ml round bottom flask containing several boiling chips. Distilled water was added to just cover the leaves and the flask enclosed in a heating mantle. A Clevenger trap was placed onto the flask and the trap was filled with distilled water. A reflux condenser was then fitted above the trap and the jacket of the condenser was cooled with circulating ice water. After 60 mins of boiling, the apparatus was cooled for 10 mins. The oil was collected in 100-µl aliquots. Such oil samples were analyzed b The A.M. Todd Company, Kalamazoo, MI 49007.

Results and discussion

In order to identify an optimum explant for *Agrobacterium* transformation, a series of preliminary experiments on shoot regeneration were carried out using rhizome, leaf or internode stem explants. Rhizomes, the most actively growing tissue, displayed the highest shoot regeneration efficiency (100% explants responded; 10–15 shoots per explant). But, due to a very high degree of internal bacterial contamination, even after numerous sterilization treatments, they were not found to be reliable material for in vitro transformation studies. Mint is known to carry a broad range of endophytic bacteria which may become immediately apparent or remain latent for long periods of time (Buckley et al. 1995). Leaf explants also had good regeneration ability, but the efficiency was lower, about 80%, and the number of shoots per explant ranged from only 1–3. Stem internode sections proved to be the best source of explants with 100% explants responding; 5–8 shoots per explant. Consequently, they were employed for *Agrobacterium* transformations. Similarly, stem internode tissue was used as the cell donor for protoplasts based on our prior experiences where an efficient protoplast-to-plant cycle was established (Krasnyanski et al. 1998).

Both the *Agrobacterium*-mediated and direct gene-transfer methods resulted in transgenic Black Mitcham plants possessing the limionene synthase gene. In our preliminary experiments internode stem explants inoculated with *Agrobacterium* strain GV3101 (pMP90) had the highest GUS-foci expression and the mean number of GUS foci per explant was significantly highest at the inoculum density of 10^5 cfu/ml (data not shown). Thus, based on these transient GUS expression studies *Agrobacterium* strain GV3101 (pMP90) at an inoculation density of 10^5 cfu/ml was employed.

In both transformation studies kanamycin was used as the selective agent at 15 mg l^{-1} in direct gene transfer and 20 mg l^{-1} in *Agrobacterium* transformation. Preliminary experiments indicated that higher kanamycin concentrations (25–100 mg l^{-1}) strongly inhibited shoot regeneration. The calli that developed from the original explants were compact, yellowish in color and, growing on the medium containing 15–20 mg l^{-1} of kanamycin, were considered resistant (putative transgenic). Most of these calli produced shoots, which were later removed, rooted, and after acclimatization in the laboratory small plants were transferred to the greenhouse. The average number of shoots per callus ranged from three to eight. At least one plant from each of 25 calli from the *Agrobacterium*

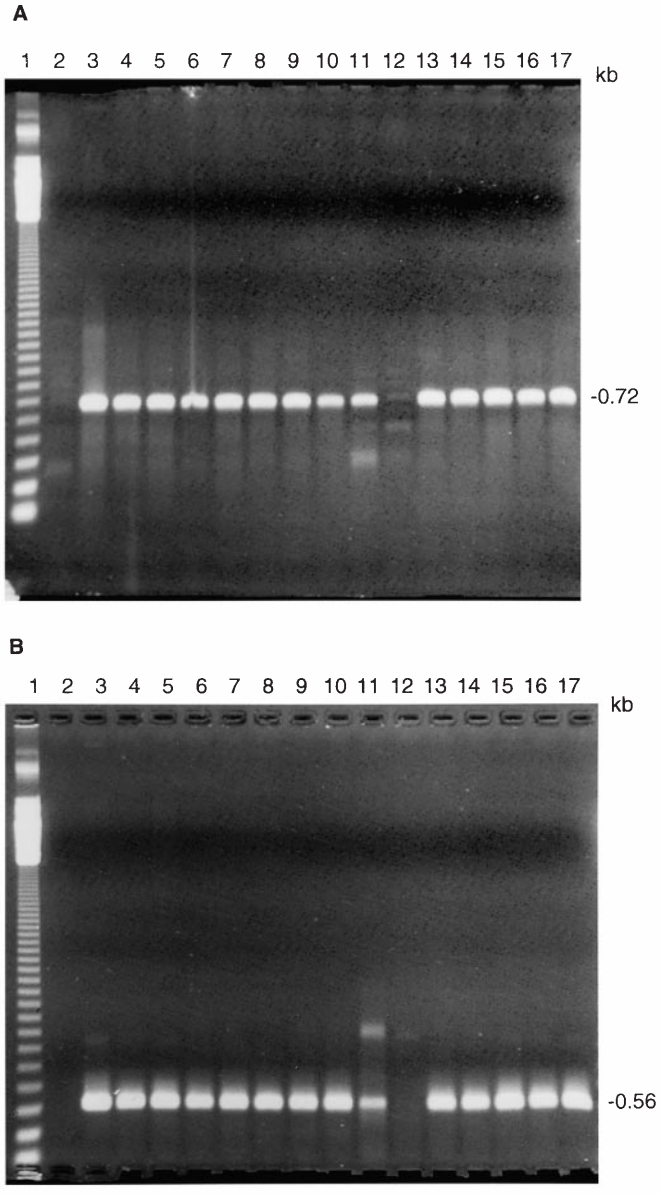

Fig. 1A, B PCR profiles of mint plants obtained after *Agrobacterium* transformation. **A** Amplification of the *npt*II gene. *Lane 1* molecular-weight-marker ladder 123; *lane 2* non-transformed Black Mitcham: *lane 3* plasmid pGALS containing the *npt*II and limonene synthase genes; *lanes 4–17* putative transgenic Black Mitcham plants; *lane 12* escape. **B** PCR-amplification of the limonene synthase gene. *Lanes* the same as in **A**

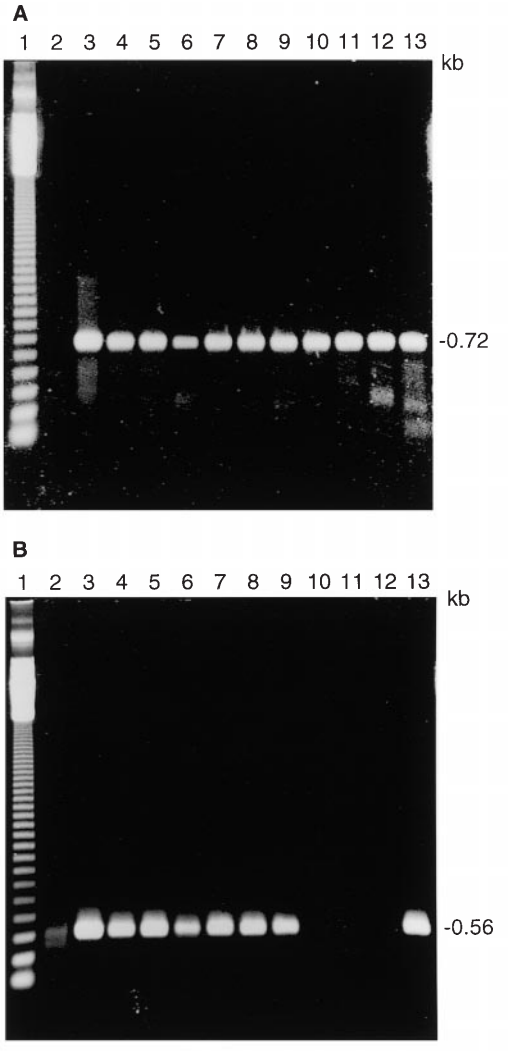

Fig. 2A, B PCR profiles of transgenic mint plants obtained after direct gene transfer. **A** Amplification of the *npt*II gene. *Lane 1* molecular-weight-marker ladder 123; *lane 2* non-transformed Black Mitcham; *lane 3* plasmid pGALS; *lanes 4–13* putative transgenic Black Mitcham plants. **B** PCR-amplification of the limonene synthase gene. *Lanes* are the same as in **A**

transformation and from the 15 calli derived from the direct gene transfer were regenerated and maintained on a pedigree basis in the greenhouse. The transgenic plants all appeared to have a typical Black Mitcham phenotype.

All regenerated plants were first screened by PCR for the presence of transgenes: *npt*II and the limonene synthase gene (Figs. 1 and 2). Since mint naturally possess the limonene synthase gene (LS), unique primers were designed for detecting the newly introduced limonene synthase. Thus, the sense primer was from the CaMV 35S promoter and the antisense primer was from the original LS gene. The results of the PCR analyses revealed that 12/25 plants regenerated after *Agrobacterium*-mediated transformation and 5/15 from the direct gene transfer did not exhibit a positive RAPD profile for both *npt*II and the LS genes (data not shown); indicating that they are escapes. We also noticed the absence of the LS gene (Fig. 2B, lanes 10–12) in some plants derived from direct gene transfer which were positive for the *npt*II gene (Fig. 2A). All positive (original plasmid's containing both *npt*II and LS genes) and negative (non-transformed mint) controls (Figs. 1 and 2) confirmed that there were no technical problems in the PCR conditions. Rearrangements that occurred following transformation using the direct gene transfer method may be the probable cause of the lack of the LS gene in some plants. However, we did not observe this phenomenon in plants regenerated from the *Agrobacterium*-mediated transformation experiments (Figs. 1A and B), although it has

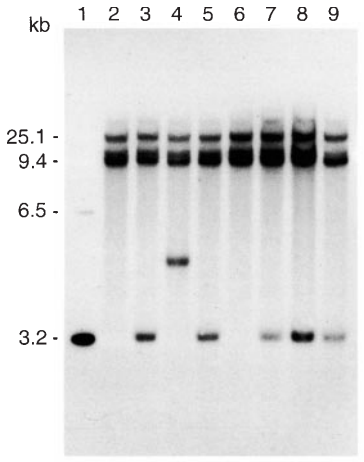

Fig. 3 Southern-blot hybridization of Black Mitcham plants transformed via *Agrobacterium. Lane 1* plasmid pGALS; *lane 2* non-transformed Black Mitcham; *lanes 3–9* transformed Black Mitcham; *lanes 3–9* transformed Black Mitcham; *lane 6* escape

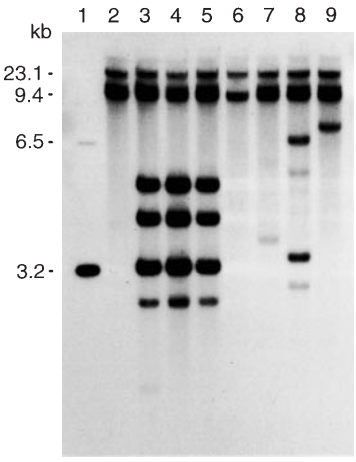

Fig. 4 Southern-blot hybridization of Black Mitcham plants transformed via direct gene transfer. *Lane 1* plasmid pGALS; *lane 2* non-transformed Black Mitcham; *lanes 3–9* transformed Black Mitcham; *lane 6* escape

been reported previously for this transformation scheme (Atkinson and Gardner 1991, 1995; Mourgues et al. 1996).

Southern-blot hybridization of seven randomly chosen putative transgenic plants (grown in the greenhouse for 6–8 months) selected from both the *Agrobacterium* transformation (Fig. 3) and the direct gene transfer studies (Fig. 4) was done with the genomic DNA digested with *Eco*RV. In both cases identical hybridization patterns, with multiple bands 10–23 kb in size, were observed. Since BM is a polyploid, the multiple bands most likely reflect copies of the natural LS gene. According to the restriction map, there are two *Eco*RV sites located downstream from and upstream of the LS gene. Thus, the restriction digest should result in the appearance of a single band, limonene synthase 3.16 kb in size, as was observed in five DNA samples from the *Agrobacterium* transformation (Fig. 3, lanes 3, 5, 7, 8, 9) and in three samples from the direct gene transfer (Fig. 4, lanes 3–5). Some LS bands were larger than 3.16 kb and of varying size, indicating independent transformation events (Fig. 4, lanes 7, 8, 9). Plants 3, 4 and 5 (Fig. 4) displayed identical hybridization patterns indicating that they are apparently from the same transformation event. In general, the direct-gene-mediated method results in more multiple and rearranged fragments in transgenic plants than does *Agrobacterium* (Dong et al. 1996), and that was the case for this study on mint. *Agrobacterium* pGA643 with LS yielded transgenic BM plants each with a single insert while 6/6 direct-mediated transgenic plants had multiple or rearranged inserts. Even though different hybridization patterns were observed, the results of Southern-blot analysis provided molecular evidence confirming the presence of the introduced DNA in the mint genome.

Fourteen transgenic single plants produced via the *Agrobacterium* (A), and likewise the ten from the protoplast (P), method were grown to 10% flowering in a greenhouse and a preliminary analysis of their essential-oil profiles by GC was conducted (Table 1). The A-transgenic plants all have low heads with the usual limonene and cineol percentages. In contrast, menthone is very high, in general, and menthol very low for most samples when contrasted to the typical mid-west piperita (MWP) sample. Thus, the menthol/menthone ratios for A-transgenics is around 0.5, much lower than the MWP of 1.8; whereas menthofuran and pulegone are very high, 2.8–27% and 1.7–16.3% respectively, compared to 1.5 and 0.6% for MWP. One A-transgenic, A9, has a unique profile with menthone (16.3.0%) and menthofuran (26.1%) considerably lower and higher, respectively, than the percentages observed for both non-transformed BM and MWP. Interestingly, A9 is the plant analyzed in lane 4, Figs. 3 that has an insert that is somewhat larger in size, about 5.0 kb than the 3.2 kb found in the other plants. Plant A23 is also unique with 41.2% menthol, much higher than the non-transformed BM and about equal to MWP. The non-transformed BM should have a profile similar to MWP, but actually has the same trends as the A- and P-transgenics when it is expected to have a more typical profile. Thus, the cause of the atypical profiles may be due to the plant maturity level or growing conditions. The P-transgenics also all have a high-menthone, low-menthol, high-menthofuran and -pugelone profile, and there are no outstanding variants in the oil profile.

These preliminary essential oil-profile data need to be interpreted with a degree of caution as it is known that while single-plant data may provide a general picture of the chemotype of a peppermint plant the ratios may not reflect analyses made by steam distillation on field plantings; that is the MWP (D. Roberts, personal communication, 1998). Significant variation occurs between hydrodistillation, used herein, and steam-distillation, and variation is also introduced by different plant cultural methods, stresses, age of planting, and maturity of the plants. Extensive field-plot studies are now required to determine both

Table 1 Essential oil profiles determined by GC analysis for single plants of Black Mitcham Peppermint, transformed by the *Agrobacterium* or the protoplast methods

Plant no.	% Essential oils							
	Head	Limonene	Cineole	Menthone	Menthofuran	Menthol	Pulegone	OL*/ONE
Agrob. (A)								
7	7.7	1.2	4.1	47.1	7.3	19.0	5.8	0.4
8	8.6	1.4	4.0	40.3	10.6	19.8	7.3	0.4
9	8.1	1.2	4.0	16.3	26.1	22.2	16.3	1.4
10	8.3	1.5	4.4	50.7	7.8	14.3	8.0	0.2
11	8.7	1.8	4.1	43.7	12.8	16.6	6.8	0.3
13	7.9	0.69	4.6	30.2	10.4	32.3	6.3	1.0
14	7.1	1.3	3.3	44.1	12.9	13.9	12.3	0.3
15	8.1	1.3	4.1	45.9	7.0	21.1	4.8	0.4
17	6.8	0.84	3.7	47.7	11.7	14.9	8.2	0.3
18	8.1	1.3	4.0	45.5	11.5	16.8	6.2	0.3
21	9.2	1.0	5.2	38.6	8.4	27.4	4.3	0.7
22	8.4	1.5	4.3	38.4	7.3	27.9	5.2	0.7
23	10.4	1.8	6.3	29.0	2.8	41.2	1.7	1.4
24	8.1	1.3	4.0	37.1	9.7	24.3	9.4	0.6
Protopl. (P)								
1	9.8	1.4	5.3	35.7	11.3	24.7	5.8	0.6
2	8.7	0.83	5.1	38.1	13.7	19.6	7.5	0.5
5	9.6	1.4	5.0	38.7	11.9	23.7	5.4	0.6
8	9.1	1.2	4.8	30.6	15.9	23.6	4.7	0.7
10	7.6	0.76	4.3	35.2	18.5	15.1	13.3	0.4
11	10.7	1.5	5.8	31.3	17.7	23.2	6.1	0.7
12	9.5	0.77	5.3	34.7	18.2	13.2	12.7	0.3
13	10.1	0.78	6.3	27.1	19.9	18.0	12.7	0.8
14	11.7	1.0	7.3	48.8	5.6	16.9	8.3	0.3
15	10.5	0.86	8.3	41.3	9.8	19.2	6.3	0.4
B.M.								
Non-trans.	8.9	1.4	4.6	47.7	9.8	16.0	4.3	0.3
Mid-west Piperita		1.2	5.9	23.0	1.5	40.8	0.68	1.8

* menthol/menthone ratio

mint oil yield and profile, and it will be of interest to follow the transgenic plants as such studies proceed.

References

An G, Ebert PR, Mitra A, Ha SB (1988) Binary vectors. A3. In: Gelvin SB, Schilperoort RA, Verma DPS (eds) Plant molecular biology manual. Kluwer Acedemic Publishers, Dordrecht, pp A-3 1–19

Atkinson RG, Gardner RC (1991) *Agrobacterium*-mediated transformation of pepino and regeneration of transgenic plants. Plant Cell Rep 10:208–212

Atkinson RG, Gardner RC (1995) Regeneration of transgenic tamarillo plants. Plant Cell Rep 12:347–351

Buckley PM, DeWilde TN, Reed BM (1995) Characterization and identification of bacteria isolates from micropropagated mint plants. In Vitro Cell Dev Biol 31:58–64

Caissard J-C, Faure O, Jullien F, Colson M, Perrin A (1996) Direct regeneration *in vitro* and transient GUS expression in *Mentha×piperita*. Plant Cell Rep 16:76–70

Chen H, Nelson RS, Sherwood JL (1994) Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. Biotechniques 16:664–670

Chilton MD, Currier TC, Farrand SK, Bendich AJ, Gordon MP, Nester EW (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. Proc Natl Acad Sci USA 71:3672–3676

Colby SM, Alonso WR, Katahira EJ, McGarvey DJ, Croteau R (1993) 4S-limonene synthase from the oil glands of spearmint (*Mentha spicata*). J Biol Chem 268:23,016—23,024

Croteau R (1991) Metabolism of monoterpenes in mint (*Mentha*) species. Planta Med Suppl 1, 57:S10–S14

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

Dong J, Teng W, Buchholz WG, Hall TC (1996) *Agrobacterium*-mediated transformation of Javanica rice. Mol Breed 2:267–276

Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13–15

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Gershenzon J, Croteau R (1991) Regulation of monoterpene biosynthesis in higher plants. In: Towers GHN, Stafford HA (eds) Biochemistry of the mevalonic acid pathway to terpenoids. Plenum Press, New York, pp 99–159

Guenther E (1972) The essential oils. Vols III—IV. RE Krieger, Huntington, New York

Helling RB, Goodman HM, Boyer HW (1974) Analysis of R *Eco*RI fragments of DNA from lamboid bacteriophages and other viruses by agarose-gel electrophoresis. J Virol 14:1235

Hokansen SC, Hancock JF, Grumet R (1997) Direct comparison of pollen-mediated movement of native and engineered genes. Euphytica 96:397–403

Kjonaas R, Croteau R (1983) Demonstration that limonene is the first cyclic intermediate in the biosynthesis of oxygenated *p*-menthane monoterpenes in *Mentha piperita* and other *Mentha* species. Arch Biochem Biophys 220:79–89

Krasnyanski S, Ball TM, Sink KC (1998) Somatic hybridization in mint: identification and characterization of *Mentha piperita* (+) *M. spicata* hybrid plants. Theor Appl Genet 96:683–687

Massimo M, Chialva F, Sacco T (1989) Glandular trichomes and essential oils in developing peppermint leaves. New Phytol 111:707–716

Mourgues F, Chevreau E, Lambert C, Bondt A (1996) Efficient *Agrobacterium*-mediated transformation and recovery of transgenic plants from pear (*Pyrus communis* L). Plant Cell Rep 16:245–249

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15:473–497

Negrutiu I, Shillito RD, Potrykus I, Biasini G, Sala F (1987) Hybrid genes in the analysis of transformation conditions. I. Setting up a simple method for direct gene transfer in plant protoplasts. Plant Mol Biol 8:363–373

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

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd edn, vol 2:9.309.62. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Spencer A, Hamill JD, Rhodes MJC (1990) Production of terpenes by differentiated shoot cultures of *Mentha citrata* transformed with *Agrobacterium tumefaciens* T37. Plant Cell Rep 8:601–604

Spencer A, Hamill JD, Rhodes MJC (1993) In vitro biosynthesis of monoterpenes by *Agrobacterium*-transformed shoot cultures of two *Mentha* species. Phytochemistry 32:911–919