A Hairy Root Culture of Melon Produces Aroma Compounds

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Musk melon is the favorite fruit with a high market value in Japan, and the fragrance is one of the major factors determining the fruit quality of melon. In this study, mutant melon hairy roots which had been induced by means of the T-DNA insertion mutagenesis were found to produce volatile compounds with the fruity fragrance of mature melon. The volatile compounds were extracted and identified by GLC−mass spectrometry. Some essential oils such as (*Z*)-3-hexenol, (*E*)-2-hexenal, 1-nonanol, and (*Z*)-6-nonenol were stably synthesized by these hairy roots despite the increased number of subcultures. The productivity of these compounds by the best hairy root line was shown to be considerably higher than naturally ripened melon fruits.

Keywords: *Melon hairy root; T-DNA insertion inactivation; Agrobacterium rhizogenes; aroma volatiles with fruity fragrance*

INTRODUCTION

Agrobacterium rhizogenes can deliver the T-DNA region of the Ri-plasmid to host plant chromosome and cause the formation of adventitious roots (hairy roots) in infected tissues as a result of expression of the *ipt* gene located on the integrated T-DNA. This bacterium-mediated gene transfer system has been mainly utilized for genetic transformation of higher plants (Tepfer, 1984) and recently applied to molecular tagging of chromosomal genes by the T-DNA insertion. The T-DNA is integrated randomly to host chromosomal regions, and therefore, this methodology would be applicable as an effective tool for producing mutants whose chromosomal genes were destroyed or inactivated by insertion with the T-DNA (Koncz et al., 1989; Hayashi et al., 1992). Using this method, we screened the T-DNA integrated mutants of hairy roots for a medical or agricultural application.

Roots of numerous plant families are the site for biosynthesis or accumulation of major secondary metabolites including alkaloids, polyacetylenes, sesquiterpenes, and naphthoquinones. These compounds could be similarly synthesized in hairy roots autonomously proliferating in the phytohormone-free medium, and their production was stable through a long-term culture, in contrast with the instability or variability commonly observed in plant cell suspension cultures (Flores et al., 1987). Our major interest is a screening and in vitro production of the substances effectively attracting insects for pest control or the flavor compounds available for industrial use. Recently, the production of essential oils was reported in anise hairy roots and their composition was compared among different tissues or organs (Santos et al., 1998). These results encouraged our group to study production of mutant hairy roots which alter the metabolism of essential oils by means of the T-DNA insertion mutagenesis. In the present study, we attempted to obtain mutant aromatic hairy roots of melon which can produce aroma volatile compounds with the fruity fragrance of melon, because melon is the favorite fruit with a high market value in Japan and the fragrance is one of the major factors determining the fruit quality of the melon.

MATERIALS AND METHODS

Hairy Root Induction and Culturing. Cotyledons of *Cucumis melo* L. (cultivar, Earl's Favorite) were inoculated with *A. rhizogenes* MAFF 03-01724 harboring the pRi1724 (Shiomi et al., 1987) with the slight modification of the previous method (Toyoda et al., 1991). Surface-sterilized seeds of melon were germinated in the dark on filter paper supplied with sterile fertilizer (1000-fold diluted Hyponex) and continuously incubated at 26 °C for 10 days under continuous illumination by a fluorescent lamp (280 μmol/m^2/S). The developed cotyledons were detached and dipped in a suspension (10^8 bacteria/mL) of *A. rhizogenes* for 10 min for inoculation. Inoculated cotyledons were transferred onto a solidified agar and incubated for 2 weeks under the light and temperature conditions mentioned above. The adventitious roots produced were excised from cotyledons and transferred onto an agar-solidified hormone-free Murashige−Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.5 g/L carbenicillin and 0.1 g/L cefotaxime and adjusted to pH 5.6. After several subcultural isolation of root tips, the bacterium-free hairy roots (fresh 2 g) were transferred to 20 mL of a liquid MS medium (without agar) and shake-cultured at 100 strokes/min for 6−9 days. Multiplied hairy roots were separated from culture solution by filtration and examined for their production of aroma volatile compounds.

On a larger scale, for liquid culture, vigorously growing hairy roots (fresh 50 g) were inoculated into 4 L of a liquid

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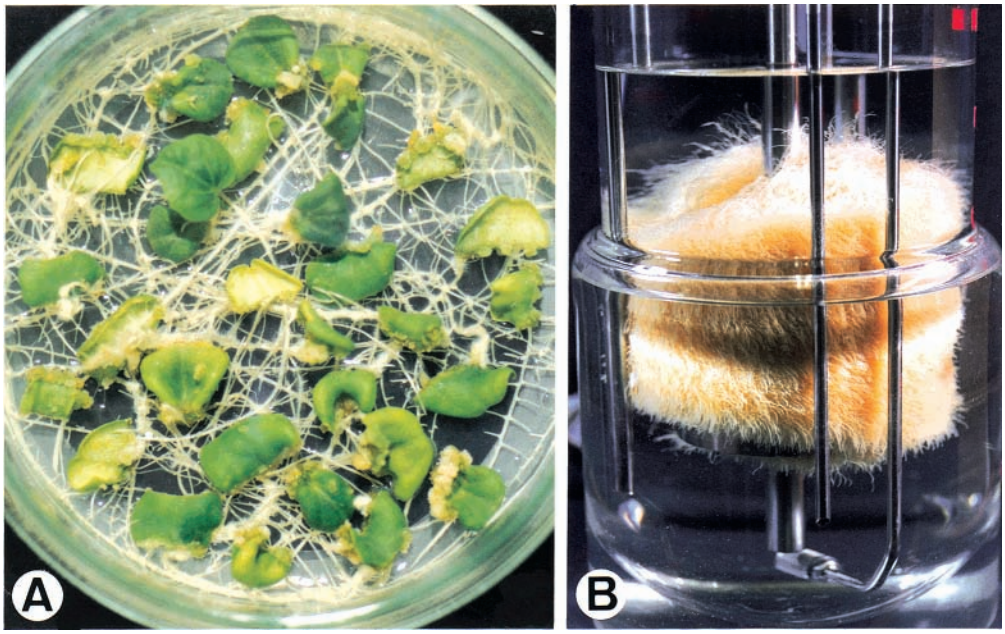

Figure 1. Induction and multiplication of hairy roots produced by melon cotyledons inoculated with *A. rhizogenes*. Numerous hairy roots were produced when cotyledons of 10-day-old seedlings were inoculated (3 weeks after incubation) (A). Vigorous multiplication of the isolated hairy root clone (KMH-009) was attained on a larger scale using a 4 L jar fermenter (2 weeks after incubation) (B).

MS medium in a jar fermenter and incubated for 2 weeks with continuous bubbling by sterile air and mechanical agitation by stirring (60 rotations/min). The medium was renewed at a 1-week interval, and the initial pH 5.6 and oxygen level (7.99 ppm at 26 °C) of the culture solution were automatically kept constant throughout the entire period of culturing.

Detection of Integrated T-DNA by PCR and Southern Hybridization. Chromosomal DNA of isolated hairy roots was extracted by the standard protocols (Murray and Thompson, 1980), and the integrated T-DNA was detected by the polymerase chain reaction (PCR), using the primers 5′-GAAGGAGTCGTGGCT-AGTTAAGTGC-3′ and 5′-AGCTACTGCCATCACTCCATTCCAAA-3′, which were artificially synthesized according to the nucleotide sequence of the *rolC* gene involved in the T-DNA of pRi1724 (Tanaka et al., 1994). The PCR was carried out with 25 cycles of the reactions, 94 °C for 1 min for denaturing, 65 °C for 1 min for annealing, and 72 °C for 1 min for extension, according to the method described previously (Matsuda et al., 1997). Alternatively, the extracted chromosomal DNA was digested with restriction endonuclease, *Bam*HI, *Nco*I, and *Xba*I, and electrophoresed for Southern hybridization. The Southern hybridization of PCR product or enzyme-digested fragments was conducted using the labeled *rolC* gene excised from the original plasmid pRi1724 as a probe.

Extraction and Identification of Volatile Compounds. Five grams of fresh hairy roots was collected by filtration, immersed in 25 mL of absolute MeOH, sealed tightly in a flask, and gently shaken at 26 °C for 24 h for extraction. The extract was filtered, and the remaining hairy roots were repeatedly extracted with the same volume of dichloromethane. All of the filtrates were combined and mixed with 50 mL of distilled water and then made alkaline by the addition of 10 mL of 5% sodium hydroxide. The dichloromethane phase collected was concentrated and subjected to GLC−mass spectrometric analysis by the previous method (Matsubara et al., 1994). The capillary GLC column DB-17 (0.25 mm × 30 m) which was linked to the mass spectrometer (Shimazu GC14BFID) was used for separation and identification of essential oils. The column was held at 50 °C for 30 min after sample injection, and then the temperature was increased at 2.5 °C/min to 280 °C.

RESULTS AND DISCUSSION

Efficient Induction of Hairy Roots and Isolation of Aromatic Mutant. Effective mutagenesis has been the most powerful tool for genetically improving a broad range of organisms. In higher plants, the Agrobacterium-mediated gene transfer could be possibly applied to the insertion inactivation of the gene and the molecular tagging of the T-DNA mutated genes (Koncz et al., 1989; Hayashi et al., 1992). *A. rhizogenes* was useful for this purpose, because of easy detection and handling of hairy roots formed as a result of the T-DNA insertion. Also in the present study, we utilized this method for genetically improving the production of essential oils in hairy roots of melon, especially aiming at the breeding of the mutant hairy root lines which could produce aroma volatiles for the fruity fragrance of melon. The efficacy of this approach, however, is dependent upon the effectiveness of hairy root initiation in inoculated plants. From this point of view, we attempted to improve our original method (Toyoda et al., 1991) and successfully attained the highly efficient production of hairy roots by the present inoculation. Numerous hairy roots (8−20 roots/cotyledon) were produced at the different sites of each cotyledon (Figure 1A), and more than 1000 hairy roots were constantly obtained in each experimental trial when this strain of *A. rhizogenes* was inoculated into detached intact cotyledons in replacement with leaf segments which were originally used for inoculation (Table 1). All of the hairy roots (total of 6543 clones) obtained were purified by a series of subcultural isolation and then olfactorily examined for their fragrance production by direct sniffing. Most of the isolated hairy roots possessed the usual rooty smell but not a fruity fragrance. As a result, we obtained five fragrant hairy root clones and eventually selected the strongest aromatic clone (KMH-009) emitting the ripe fresh fruity fragrance of melon. Thus, the present method for inoculation was effective enough to obtain mutant hairy roots of melon.

Table 1. Induction of Hairy Roots (HRT) by Melon Cotyledons Inoculated with A. rhizogenes and Selection of Fragrant Hairy Roots

experiments	inoculated cotyledons	HRT-producing cotyledons	total HRT	fragrant HRT
1	100	98	1050	1
2	121	117	1420	2
3	123	116	1450	0
4	108	99	1313	1
5	117	112	1310	1
total	569	542 (95.3)[a]	6543	5
control[b]	108	12 (11.1)	23	0

[a] Percentage of hairy-root-inducing cotyledons. [b] Segments of young leaves of 1-month-old seedlings were inoculated according to the previous method (Toyoda et al., 1991).

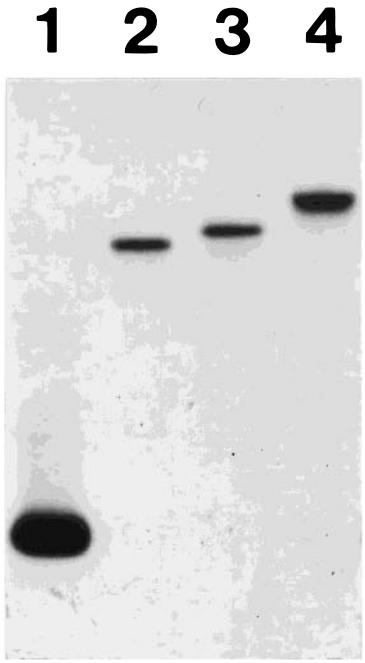

Figure 2. Southern hybridization detection of the *rolC* gene integrated into the chromosome of melon hairy root clone KMH-009. Lane 1 represents the positively hybridized fragment amplified by PCR, and lanes 2−4 show the single positive fragment generated by the digestion with *Bam*HI, *Nco*I, and *Xba*I, respectively. The labeled *rolC* gene was used as probe for hybridization. The ladder shows the size-marker provided by *Hind*III-digested λ DNA.

Successful Integration of T-DNA. Successful integration of the T-DNA into chromosomal DNA of the KMH-009 was first examined by PCR-amplifying the *rolC* gene located on the integrated T-DNA. The results indicated that the hybridization-positive DNA fragment with the expected length (0.98 kb) was specifically amplified by the PCR primers designed in the present study (lane 1 in Figure 2). Secondarily, the chromosomal DNA was digested with some restriction endonucleases which possessed their own unique cleavage sites at the outer regions of the *rolC* in the T-DNA (Tanaka et al., 1994) in order to determine the copy number of the integrated T-DNA by a Southern hybridization analysis. Consequently, the positive band was detected singly at the 12.0, 14.0, and 18.0 kb positions in the *Bam*HI, *Nco*I, and *Xba*I digestion, respectively (lanes 2−4 in Figure 2), suggesting that a single copy of T-DNA was integrated into the chromosome of the KMH-009.

Identification of Aroma Volatiles and Stable Production. The volatile compounds were extracted from the KMH-009 hairy roots and identified by the GLC-MS spectrometer (Table 2). In this study, the hairy roots exponentially proliferating in a hormone-free liquid medium were used for extraction. In addition, the nonfragrant hairy root clone (KMH-028) was used for comparison. The data showed that the aromatic hairy roots could newly synthesize some essential oils such as (*Z*)-3-hexenol, (*E*)-2-hexenal, 1-nonanol, and (*Z*)-6-nonenol, which were reported to be important aroma volatiles in melon (Buttery et al., 1982). Of these compounds, (*Z*)-6-nonenol provides the strong fruity fragrance of ripe fresh fruits of melon at low concentrations (Buttery et al., 1982). These results strongly suggest that the hairy roots of the KMH-009 would be aromatized by the synthesis of these essential oils. On the contrary, the culture filtrate was not fragrant and the notable aroma volatiles were not detected in the culture solution separated from the hairy roots, indicating that these compounds were not secreted or released into the culture medium.

Table 2. Percentage Composition of Essential Oils from Fragrant Hairy Root Clones of Melon

		hairy root clones[b]			
		009		022	028[c]
R.T.[a]	essential oils identified by GLC-MS	HRT	CF	HRT	HRT
4.55	2-hexanal	6.58	18.38	1.85	9.00
4.81	ethyl 2-methyl butyrate	1.86	6.28	1.39	2.32
5.16	ethyl butylate	3.95		2.19	4.19
5.50	isoamyl alcohol	4.55		2.72	4.70
7.70	toluene	1.98		1.65	1.34
8.34	1-hexanal	0.85		1.43	
11.42	1-hexanol	0.17		0.11	
11.64	(*Z*)-3-hexenol	0.13		3.02	
12.23	3-methyl butyl acetate	44.41	7.67	26.93	26.08
12.71	(*E*)-2-hexenal	9.56		7.18	
20.79	ethyl hexanoate	1.74		1.83	2.30
22.25	1-nonanal	11.52		4.61	7.99
24.19	(*E*,*Z*)-2,4-heptadienal	0.21		0.21	2.32
24.58	benzyl aldehyde	0.28		1.38	
25.39	(*E*,*E*)-2,4-heptadienal	0.11		0.14	
30.30	benzyl alcohol	3.51		16.78	2.16
32.46	1-nonanol	0.72		1.62	
34.09	(*Z*)-6-nonenol	0.14		0.22	
34.46	(*E*,*Z*)-2,6-nonadienal	0.03		0.29	
35.94	phenyl ethyl alcohol	0.23		2.25	0.89
	others	7.47	67.67	22.20	36.71
	total extract (mg)	12.3	7.4	13.2	11.7

[a] Retention times (min) on a DB-17 capillary column. [b] Five grams of hairy roots (HRT) and 20 mL of culture filtrate (CF) were separately used for extraction. [c] Nonfragrant clone as control.

The stable production of the fruity aroma volatiles by the KMH-009 was assessed by comparing the yields of the compounds from the hairy roots repeatedly subcultured for more than 3 years. The data revealed that the essential oils for aroma scent were constantly synthesized with no relation to the increased number of times for subculture, and the constant production by this clone was successfully maintained in the hairy roots repeatedly subcultured (Table 3).

In an intact melon plant, the fruity aroma volatiles were synthesized mainly at the stage of maturation or ripening of fruits, thereby suggesting that the expression of the genes involved in the biosynthesis of these essential oils would be controlled organ- and differentiation-specifically by the regulatory regions of the chromosome. Thus, we speculate that the KMH-009 was a mutant hairy clone line constitutively synthesizing aroma volatile compounds as a result of destroying such a regulatory region by the T-DNA insertion.

Table 3. Stable Production of Fragrant Volatiles by the Hairy Root Clone KMH-009 of Melon

	percentage			
aroma essential oils	5	50	92	144[a]
(*Z*)-3-hexenol	0.12	0.12	0.13	0.24
(*E*)-2-hexenal	9.22	8.29	9.56	8.17
1-nonanol	0.81	0.84	0.72	0.64
(*Z*)-6-nonenol	0.15	0.15	0.14	0.16
total extracts (mg)[b]	11.4	11.8	12.3	13.4

[a] The number of times for subculture repeated at 6-day intervals. [b] From 5 g of fresh hairy roots exponentially proliferated.

Table 4. Comparison of Productivity of Major Aroma Volatiles between KMH-009 Hairy Roots and Fresh Ripe Fruit of Melon

	total yields (mg)[a]	
aroma essential oils	hairy roots	fruit sarcocarp
(*Z*)-3-hexenol	5.14	trace
(*E*)-2-hexenal	217.31	66.20
1-nonanol	4.18	trace
(*Z*)-6-nonenol	8.43	1.31

[a] Sarcocarp (approximately 400 g) of a single melon fruit and the same weight of large-scale cultured hairy roots were used for extraction.

Preliminary Approach of Scale-up Production of Aroma Volatiles. The present study demonstrated that the capability of the mutant clone to produce aroma compounds could be successfully transferred to vegetatively propagated hairy roots through a long-term culture. These results prompted us to apply the KMH-009 hairy roots to large-scale production of aroma volatiles, for a practical application to the industrial and agricultural use. In the present study, the volume of culture medium was 200-fold scaled up using a jar-fermenter system. Eventually we obtained the bulk of multiplied hairy roots (approximately 400 g fresh weight) after 2 weeks of incubation (Figure 1B). The overall profile of the extracted essential oils was completely similar to that of hairy roots cultured on a routine laboratory scale (data not shown). Also, there was no considerable difference in the quantity of total extracts between the routine-scale (2.8 ± 0.3 mg/g.fr.wt.) and the scale-up cultured hairy roots (2.5 ± 0.2 mg/g.fr.wt.). The increased yields of aroma volatile compounds could be archived by optimizing both the culture condition permitting active growth of the hairy roots within shorter periods of incubation and the procedure for large-scale extraction.

In relation to the aroma essential oils produced by the KMH-009, the productivity was compared with fresh ripe fruits of melon cultivar from which the hairy roots were originally generated. The analytical data revealed that the KMH-009 showed the higher production of these compounds than those of sarcocarp of melon fruits cultivated under an authenticated condition, approximately 6.5-fold higher especially in (*Z*)-6-nonenol (Table 4).

Melon is the favorite fruit with a high market value in Japan, and the fragrance is one of the major factors to determining the fruit quality of melon. In general, the mellow fragrance is known to be produced by high-quality and high-price netted musk melons, and therefore, various cultivars or lines of netted musk melon have been bred in our university farm in order to improve fragrance characteristics as well as tasty quality. The melon cultivar used in the present study is a hybrid progeny between two lines (Haru 2 and Haru 3) of Earl's Favorite and gives the most profitable fragrance to fresh ripe fruits of melon grown by the authenticated cultivation. However, the direct extraction of aroma volatile compounds has been impractical because of the high cost and long period of fruit production. On the contrary, the present results can emphasize some advantageous features of the in vitro utilization of mutant hairy roots: (i) easy handling of cultured hairy roots, (ii) possible application of molecular biological and tissue culture techniques to genetic improvement of hairy roots, (iii) constant growth and productivity of hairy roots, (iv) shorter periods of culture in limited space, and (v) controlled scale-up for industrial use. Recently, we successfully obtained the additional mutant clone (KMH-022) of hairy roots with a higher production of (*Z*)-3-hexenol, 1-nonanol, and (*Z*)-6-nonenol (Table 2). The selection for the more profitable mutant lines would be useful for the breeding or improvement of hairy roots and for the more efficient in vitro production of plant secondary metabolites including aroma essential oils.

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