Genetic Analysis of Nothoapiol Formation in *Perilla frutescens*

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A new essential oil chemotype of *Perilla frutescens* was found in that plant variety from Che-ju island in Korea. The steam-distilled oil of this plant was examined by GC-MS and 32 compounds were identified. Its principal constituents were β -caryophyllene, dillapiol and nothoapiol. Also, crossing experiments were performed between the new chemotype and known chemotypes to clarify the genetic control of the production of nothoapiol. The gene *Na*, which promotes conversion from dillapiol to nothoapiol, was suggested, and was considered to be closely linked with the allele *D*.

Key words *Perilla frutescens*; nothoapiol; genetic control; essential oil; GC-MS

Our previous studies revealed several chemotypes of essential oil composition in *Perilla frutescens* and also various patterns of genetic control in the expression of these chemotypes.^{1–8)} The oil types of *P. frutescens* are roughly divided into two categories according to the principal constituents: *i.e.* the monoterpene (Mt) type, which principally contains monoterpene compounds but few phenylpropanoids in the leaves, and the phenylpropanoid (Pp) type containing large amounts of phenylpropanoids but few monoterpenoids. Four subtypes are known within the Pp type: *i.e.* myristicin (M) type, elemicin/myristicin (EM) type, dillapiol/myristicin (DM) type and the dillapiol/elemicin/myristicin (DEM) type. Recently, a new subtype was discovered among the Pp type strains collected from the Korean island Che-ju, and the essential oil of this new chemotype (strain No. 5316) was extracted by steam-distillation and analyzed by GC-MS. Crossing experiments were also performed between this new chemotype and known Pp subtypes to clarify its pattern of genetic control.

MATERIALS AND METHODS

Plant Materials and Crossing Experiment The strains of *P. frutescens* used in this study were No. 12 (M type), No. 10 (EM type), No. 16 (DM type), No. 25 (DEM type), No. 5254 (citraol (C) type) and No. 5316 (NDM type), all of which had been propagated by self-pollination for several years to ascertain that no genetic segregation for the composition of the essential oil occurred. Fresh leaves of the perilla strains grown in the field of the Experimental Station for Medicinal Plants, Graduate School of Pharmaceutical Sciences, Kyoto University (ESM) were collected in September for GC-MS analysis of the essential oils. Voucher specimens were kept in the herbarium of ESM. The methods and procedures of the artificial crossing were described previously.¹⁾

GC-MS Analysis of the Essential Oil Composition of Strain No. 5316 Fresh leaves (*ca.* 200 g) were steam-distilled with 750 ml of ion-exchanged water for 2 h using the apparatus designated by the Japanese Pharmacopoeia XIII.⁹⁾ The extracted oil was preserved in an airtight container at -30°C before analysis. GC-MS analysis was performed on a Hewlett Packard 5890 series II gas chromatograph connected to an Automass 50 (JEOL). The operation conditions were as follows; column: fused silica capillary column, TC-wax (Hewlett Packard), 60 m $\times$ 0.25 mm, film thickness=0.25 μm ;

column temperature: 100–200 $^{\circ}\text{C}$ increasing at 3 $^{\circ}\text{C}/\text{min}$, ending at 200 $^{\circ}\text{C}$ for 20 min; injector: 180 $^{\circ}\text{C}$; carrier gas: helium at a flow rate of 30 cm/s; column head pressure: 180 kPa; injection volume: 0.5 ml; ionization energy: 70 eV; ion source temperature: 200 $^{\circ}\text{C}$. Chemical components were identified by comparing their retention times and mass spectra with those of authentic samples and/or reference data,^{1,10)} or by comparing their mass spectra with those in the MS data library (NBS library). The relative amount of each component was determined by calculating the peak area of the TIC chromatogram.

Determination of the Essential Oil Type a) **TLC Analysis:** Fresh leaves (*ca.* 1 g) were crushed in 1 ml of diethyl ether and the extract was applied to a TLC plate (Kieselgel 60, Merck), developed with a mixture of benzene-ethyl acetate=19:1, then heated to color after spraying with anisaldehyde- H_2SO_4 . The chemotypes of F_1 and F_2 progeny were determined by comparing their TLC patterns with those of authentic samples and/or those of parental strains. The chemotypes were confirmed by GC analyses.

b) **GC Analysis:** Samples of 1 g of fresh leaves were extracted with 1 ml of diethyl ether overnight at 4 $^{\circ}\text{C}$, then dehydrated with Na_2SO_4 , and the resultant ether fraction was subjected to chromatography on a Hitachi G-5000 under the following operation conditions; column: glass column (3 m $\times$ 2 mm) filled with OV-17 Shimadex W, 3% (80–100 mesh, Shimadex); column temperature: 190 $^{\circ}\text{C}$ (for phenyl propanoid type), 160 $^{\circ}\text{C}$ (for monoterpene type); injector: 180 $^{\circ}\text{C}$; detector: 180 $^{\circ}\text{C}$, hydrogen flame ionization detector (FID); carrier gas: nitrogen at a flow rate of 24.5 ml/min; column head pressure: 70 kPa.

RESULTS AND DISCUSSION

GC-MS Analysis of the Essential Oil The results of GC-MS analysis of the essential oil of No. 5316 extracted by steam-distillation are shown in Table 1. Thirty-two compounds were identified, including several monoterpene compounds such as perillene, linalool, shisofuran¹¹⁾ and perillaketone, as well as the principal phenylpropanoids: myristicin, dillapiol and nothoapiol. However, the relative amounts of these phenylpropanoids were considerably different from those calculated from the results of analysis by GC with a glass column. These differences were supposed to be due to differences in the genetic control of trichomes on phyllo-

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planes compared with those on petioles, stems or veins (unpublished data); thus, although phylloplanes of No. 5316 contain Pp type compounds, its petioles and veins were considered to contain Mt type compounds. The samples for GC analysis were only 1 g each, and therefore we could carefully remove the petioles. On the other hand, samples of 200 g

were used for GC-MS analysis and hence they contained non-negligible amounts of veins and petioles.

Crossing Experiments The principal constituents in the essential oil of the parental strains used in this study are listed in Table 2. The relative amounts of myristicin, dillapiol and nothapiol in the essential oil of No. 5316 determined by GC equipped with a glass column were 14%, 38% and 48%, respectively, thus, we classified this strain as the NDM type. Although β -caryophyllene is one of the principal constituents in the essential oil of perilla, this compound has little significance in the genetics of essential oil compounds and is contained in all chemotypes. The results of crossing experiments and the possible genotypes of the parental strains derived from the results are shown in Tables 3 and 4.

(1) NDM $\times$ DM: The essential oil type of the F_1 hybrid between No. 5316 (NDM type) and No. 16 (DM type) was the NDM type and gave a 3:1 segregation ratio between the NDM type and the DM type in F_2 progeny (Table 3). Nothapiol was supposed to be biosynthesized from dillapiol by methyl-etherealization of the remaining hydrogen residue. Therefore, the resultant segregation ratio can be explained by postulating a dominant allele, designated *Na* which promotes the conversion from dillapiol to nothapiol (Fig. 1). As described previously, No. 16 was homozygously dominant (*DD*) for the gene which controls the reaction step from myristicin to dillapiol.³⁾ Thus, the segregation ratio of the present results suggested that the genotypic difference between the NDM and DM types concerns only one dominant allele, *Na*. The genotypes of No. 16 and No. 5316 can therefore be expressed as *DDnana* and *DDNaNa*, respectively.

(2) M $\times$ NDM: In the cross between No. 12 (M type) and No. 5316 (NDM type), the F_1 plants showed the NDM phenotype, and the F_2 progeny were segregated in a ratio of 3:1 between the NDM type and the M type (Table 3). Assuming that the genes *Na* and *D* were inherited independently of each other, and that the genotype of No. 12 was *ddnana*, the segregation ratio in F_2 progeny should be 9:3:4 for the NDM type:DM type:M type. Nevertheless, the observed ratio was 3:1 for NDM type:M type, while none of the DM type was

Table 1. Composition of the Essential Oil of No. 5316

	Compound	Content (%)
1	Hexanal	0.17
2	β -Pinene	tr
3	Limonene	tr
4	(<i>E</i>)-2-Hexenal	0.41
5	1-Methylbutyl oxirane	tr
6	3-Octanol	0.42
7	2-Hexene-1-ol	tr
8	Perillene	0.26
9	1-Octen-3-ol	5.12
10	Ylangene	tr
11	Linalool	0.20
12	Benzaldehyde	0.40
13	Elemene	tr
14	β -Caryophyllene	31.87
15	Shisofurane	tr
16	β -Farnesene	1.60
17	Isocaryophyllene	2.69
18	Germacrene D	0.74
19	α -Farnesene	10.15
20	Naphthalene	0.54
21	δ -Cadinene	tr
22	Perillaketone	4.21
23	Geraniol	tr
24	Patchoulene	tr
25	Caryophyllene oxide	tr
26	Nerolidol	0.31
27	Isoeugenol	0.13
28	1,2,3,4-Tetramethoxy-5-(2-propenyl)-benzene	0.43
29	Elemicin	0.13
30	Myristicin	4.30
31	Dillapiol	21.13
32	Nothapiol	13.51

Table 2. Principal Constituents in the Essential Oils of Parental Strains

Strain	Chemotype	C	PL	PK	M	D	E	N
5316	NDM	—	—	—	+	++	—	++
16	DM	—	—	—	+	++	—	—
10	EM	—	—	—	+	—	++	—
25	DEM	—	—	—	+	+	++	—
12	M	—	—	—	++	—	—	—
5254	C	++	+	+	—	—	—	—

++, major compound; +, minor compound; —, not detected; C, citral; PL, perillene; PK, perillaketone; M, myristicin; D, dillapiol; E, elemicin; N, nothapiol.

Table 4. Proposed Genotypes of *Perilla* Strains Used in This Study

Strain	Chemotype	Genotype
5254	C	<i>GGhhnnfr₁fr₂fr₂jjDDNaNaEE</i>
12	M	<i>ggddnanaee</i>
16	DM	<i>ggDDnanaee</i>
10	EM	<i>ggddnanaEE</i>
25	DEM	<i>ggDDnanaEE</i>
5316	NDM	<i>gghhnnFr₁Fr₁Fr₂Fr₂JJDDNaNaee</i>

Table 3. Segregation of the Essential Oil Types in F_2 Progeny of Crosses between Different Oil Types of *Perilla frutescens*

Crossing $P_1 \times P_2$ ($\sigma \times \sigma$)	F_1 Chemotype	F_2			
		Chemotype	Observed ratio	Expected ratio	<i>p</i> value of χ^2 test
5316 $\times$ 16 (NDM $\times$ DM)	NDM	NDM:DM	56:15	3:1	>0.3
12 $\times$ 5316 (M $\times$ NDM)	NDM	NDM:M	52:18	3:1	>0.8
10 $\times$ 5316 (EM $\times$ NDM)	NDEM	NDEM:NDM:EM:M	64:18:17:5	9:3:3:1	>0.7
5316 $\times$ 25 (NDM $\times$ DEM)	NDEM	NDEM:NDM:DEM:DM	37:16:10:3	9:3:3:1	>0.5
5316 $\times$ 5254 (NDM $\times$ C)	PK	PK:PL:C:NDEM:NDM	61:12:6:28:5	135:45:12:48:16	>0.2

Fig. 1. Possible Reaction Steps Controlled by the Alleles G , E , D and Na in the Hypothetical Biosynthetic Pathway

➡, promotion; ⇨, inhibition.

observed. This result could be interpreted in two ways; No. 12 may be homozygously recessive for the gene Na , and this gene may be closely linked with D . Therefore, the genotype of No. 12 would be expressed as $ddnana$ and that of No. 5316 would be $DDNaNa$. Alternatively, the genotype of No. 12 may not be $ddnana$ but $ddNaNa$, and hence the genotype of No. 5316 would be expressed as $DDNaNa$. Yet, the results of the crossing experiments of $M \times NDM$ could not conclusively determine the genotypes of these strains.

(3) **EM $\times$ NDM:** The chemotype of the F_1 plant obtained by crossing No. 10 (EM type) and No. 5316 (NDM type) was the NDEM type, and the segregation ratio of the F_2 progeny was 9:3:3:1 for NDEM type:NDM type:EM type:M type (Table 3). This observed ratio indicated that two alleles were involved with the segregation of the F_2 progeny of this cross: the gene D and gene E which were demonstrated previously to be independent of each other.³⁾ Strain No. 10 has previously been shown to generate no nothoapiol-containing type in its progeny through crosses with the DM or DEM type,³⁾ and none of the DEM type was observed among the F_2 progeny of these crosses, hence, No. 10 was supposed to be homozygously recessive for the gene Na . Accordingly, No. 10 was homozygously recessive for allele D and allele Na but was dominant for allele E , and No. 5316 was homozygously dominant for both allele D and allele Na but recessive for allele E , which suggests that allele Na is linked with allele D . Thus, the genotypes of No. 5316 and No. 10 are expressed as $DDNaNaee$, $ddnanaEE$ respectively. Those of the NDEM type, the NDM type, the EM type and the M type in the F_2 progeny are expressed as $D-Na-E-$, $D-Na-ee$, $ddnanaE-$ and $ddnanaee$, where, D -stands for DD and Dd . These results suggest that the genotype of No. 12 is $ddnana$.

(4) **NDM $\times$ DEM:** The cross between No. 5316 (NDM type) and No. 25 (DEM type) generated the NDEM type as the F_1 progeny, which gave a 9:3:3:1 segregation ratio for NDEM type:NDM type:DEM type:DM type in the F_2 progeny. This observed ratio indicated that the segregation was due to two alleles, E and Na . Accordingly, the genotypes of No. 5316 and No. 25 were described as $DDNaNaee$ and $DDnanaEE$ respectively, and those of the F_2 progeny were $DDNa-E-$, $DDNa-ee$, $DDnanaE-$ and $DDnanaee$, respectively, for the NDEM type, NDM type, DEM type and DM type.

(5) **NDM $\times$ C:** The control system for production switching between the Mt type and the Pp type constituents is illustrated by postulating the gene G .^{2,12)} The chemotype of the F_1 hybrid between No. 5316 (NDM type) and No. 5254 (C type) was the PK type, and the segregation ratio of the F_2 progeny was 135:45:12:48:16 for PK type:PL type:C type:NDEM type:NDM type, respectively. The PK, PL and C types are classified as belonging to the Mt type, and the NDEM and NDM types are classified as the Pp type, thus, the segregation ratio between the Mt type and the Pp type was calculated to be 3:1, indicating that the control system of the gene G is applicable to the NDM type. The PK type and the PL type were observed in the F_2 progeny, however, since No. 5254 is homozygously recessive for the polymeric genes Fr_1 and Fr_2 which promote the conversion from citral to perillene,⁸⁾ and is also recessive for the gene J promoting the conversion from perillene to egomaketone,⁵⁾ the genes Fr_1 , Fr_2 and J in F_2 plants were presumed to be inherited from No. 5316. That is, No. 5316 was presumed to possess these genes in a dominant form. On the other hand, neither the PA type nor the EK type were observed among the F_2 progeny; hence, No. 5316 was homozygously recessive for the gene H ²⁾ which promotes the formation of limonene, and it is also recessive for the gene N ¹²⁾ which promotes the conversion from citral to nagnataketone. The genotypes of No. 5316 and No. 5254 can thus be expressed as $gghhnnFr_1Fr_1Fr_2Fr_2JJDDNaNaee$, and $GGhhnnfr_1fr_1fr_2fr_2jjDDNaNaEE$, respectively.

Revised Relationship between the Genotype and Myristicin Content:

The relative amount of myristicin detected in the NDM and NDEM types among the F_2 progeny resulting from crossing No. 12 (M type) $\times$ No. 5316 (NDM type) and No. 10 (EM type) $\times$ No. 5316 varied from trace to 26.3%; the higher content progeny showed 5.8–26.3%, while the lower content progeny showed trace–2.2%. The content of myristicin was reported to be determined by the zygosity of the two genes D and E ,³⁾ however, F_1 progeny of both crosses of No. 12 $\times$ No. 5316 and No. 10 $\times$ No. 5316 were rich in myristicin (21.75, 15.56%) although their genotypes were $DdEe$, and $Ddee$ respectively. The content of elemicin in the NDEM type ($DDE-$, 16.69–53.92%) and in the EM type ($ddE-$, 22.24–63.13%) among the F_2 progeny were almost the same, but those in the NDM type ($D-ee$) were varied. These results indicated that the myristicin content is principally determined by the zygosity of the gene D ; homozygosity for the gene D (DD , dd) resulted in myristicinless plants whereas heterozygosity for this gene (Dd) resulted in myristicin-rich plants.

The series of subtypes of the Pp type in *Perilla*, which are genetically well-studied in reference to its regulation system,

would serve as good plant materials for studying the biosynthetic pathway of phenylpropanoids. Many of the essential oil constituents of C₆-C₃ show pharmacological activities which make them promising for further examination.

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