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GENETIC CONTROL OF THE CHEMICAL COMPOSITION OF VOLATILE OILS IN *PERILLA FRUTESCENS*

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Key Word Index—*Perilla frutescens*; Labiatae; volatile oil; chemotype; gene analysis; hybridization; multiple alleles.

Abstract—In order to clarify the genetic basis for chemical variations of the volatile oil components in *Perilla frutescens*, the F_1 and F_2 progenies of the artificial hybrids between the following different chemotypes have been examined individually for chemical compositions of the volatile oils extracted from the leaves: PA (perillaldehyde), EK (elsholtziaketone), PK (perillaketone) and PP (phenylpropanoid) types. The results of the genetic experiments have clearly demonstrated that the chemical composition is controlled by a series of multiple alleles (G_1, G_2, g) and an independent pair of alleles (H, h). The presence of either G_1 or G_2 is essential for initiating the biosynthesis of any monoterpenoids in *Perilla* plants. However, G_1 and G_2 function differently in determining the kinds of monoterpenes to be produced; G_1 that produces the EK type is dominant over G_2 that produces the PK type, while the homozygous recessive (gg) plants produce characteristic phenylpropanoids (myristicin, dillapiol, elemicin) in place of the monoterpenoids. In the presence of G_1 or G_2, plants homozygous for the dominant allele H (G-HH) produce volatile oil of the PA type, whereas those heterozygous for H (G-Hh) accumulate a large amount of L-limonene which is considered to be an intermediate metabolite in the biosynthesis of perillaldehyde. The roles of these genes are discussed in relation to the biosynthetic pathways.

INTRODUCTION

Perilla frutescens, an annual herb cultivated extensively in Japan both as a spice and a crude drug, consists of several chemical verieties which differ in the constituents of the volatile oils in leaves. Ito [1] has classified these varieties into four chemotypes on the basis of the main components of the volatile oils, viz. perillaldehyde (PA), furylketone (FK), citral (C) and phenylpropanoid (PP) types, while Nagao *et al.* [2] have reported the existence of PA, PP, perillaketone (PK) and elsholtziaketone (EK) types in a collection of local varieties or strains. Recently, we have carried out GC analysis of the volatile oils of 215 strains of *Perilla* collected from various parts of Japan to group them into five distinct types: PA, EK, PK, C and PP [3]. These chemotypes proved to be genetically stable, showing no segregation for the chemical composition in the self-pollinated progenies. However, a newly found 'LPA type' that contains L-limonene as a main component and perillaldehyde as a minor one, has been shown to be a natural hybrid. To elucidate the genetic mechanism for the chemical differences in volatile oils, we undertook breeding experiments with these five chemotypes. The present paper reports for the first time that the chemical diversity is caused by two pairs of independent genes. Also, the validity of the hypothetical biosynthetic pathways leading to various components of *Perilla* oil is re-examined in view of the genetic data.

RESULTS

From the results of GC analysis, the parental strains used for the cross experiments were classified into four chemotypes according to the major components of volatile oils (Table 1). These chemotypes can be clearly distinguished by their characteristic components; the PA (perillaldehyde) type containing perillaldehyde and L-limonene as the two major components, the EK (elsholtziaketone) type containing mainly elsholtziaketone with naginataketone as a minor component, the PK (perillaketone) type containing perillaketone and a small amount of isoegomaketone and the PP (phenylpropanoid) type with little or no monoterpenoids but phenylpropanoids (myristicin, dillapiol and elemicin).

Intercrosses were made between the four chemotypes PA, PK, EK and PP. Table 2 shows the F_1 and F_2 data obtained from these intercrosses.

PP × PA

In the crosses between PP and PA strains, the phenotypes of both F_1 and F_2 plants varied with the parental strains. In the case of 16 (PP) × 75 (PA), the F_1 plant was of the PA type which gave, in the F_2 generation, PA and PP type plants in a 3:1 ratio. On the other hand, the cross 5 (PP) × 9 (PA) or 12 (PP) × 32 (PA) gave F_1 plants of the LPA type containing L-limonene as a major component and perillaldehyde as a minor one of the volatile oil. In F_2, four different types PA, LPA, PK and PP appeared in a ratio of 3:6:3:4. These results could be tentatively explained by assuming that all the PA strains (9, 32, 75) were homozygous for two pairs of dominant alleles (GGHH), whereas the genotypes of the PP strains were either ggHH (strain 16) or gghh (strains 5 and 12). Here the presence of a dominant allele G_1 or G_2 is considered to be essential for the production of any monoterpenes, and the phenotypes PA, LPA, and PK would be expressed by

Table 1. Main components of the volatile oils in the parental strains of *Perilla frutescens* used for intercrosses

Chemotype	Strain	Main components of volatile oils								
		LI	PA	EK	NK	PK	IK	MY	DI	EL
PA	9, 32, 75, 76	+	++	−	−	−	−	−	−	−
EK	3, 79	−	−	++	+	−	−	−	−	−
PK	6, 8, 11	−	−	−	−	++	+	−	−	−
PP	1, 5, 12	−	−	−	−	−	−	++	−	−
	16	−	−	−	−	−	−	+	++	−
	70	−	−	−	−	−	−	+	−	++

++, Major compound; +, minor compound; −, not detected; LI: L-limonene; PA, perillaldehyde; EK, elsholtziaketone; NK, naginataketone; PK, perillaketone; IK, isoegomaketone; MY, myristicin; DI, dillapiol; EL, elemicin.

Table 2. Genetic segregations in the F_2 progenies of the intercrosses between *Perilla* strains showing different chemotypes

Cross ($P_1 \times P_2$)*		Phenotype of F_1	Genetic segregation of F_2			
			Phenotype	Observed ratio	Expected ratio	P value of χ^2 test
PP × PA	16 × 75	PA	PA:PP	41:11	3:1	0.8
PP × PA	5 × 9	LPA	PA:LPA:PK:PP	9:29:9:13	3:6:3:4	0.3
	12 × 32			5:6:5:5		0.7
PK × PA	11 × 32	LPA	PA:LPA:PK	14:32:9	1:2:1	0.2
	8 × 75			14:26:13		0.9
PK × PP	8 × 16	LPA	PA:LPA:PK:PP	1:16:7:9		0.06
	8 × 70			12:12:6:4		0.2
PP × PK	16 × 6			3:13:4:8		0.5
PK × EK	8 × 79	EK	EK:PK	36:6	3:1	0.1
PP × EK	1 × 3	LPA	PA:LPA:EK:PP	27:44:19:26	3:6:3:4	0.5
	16 × 79			5:8:2:4		0.6
PA × EK	76 × 79	LPA	PA:LPA:EK	13:25:13	1:2:1	0.9

*P_1, Female parent; P_2, male parent.

the genotypes G-HH, G-Hh, and G-hh, respectively, where G- stands for either GG or Gg.

PK × PA

The F_1 hybrids of this cross (11 × 32, 8 × 75) were also of the LPA type and gave PA, LPA, and PK plants in a 1:2:1 ratio in F_2. This segregation ratio is expected from the above assumption that the genotypes of PK and PA parents were GGhh and GGHH, respectively.

PP × PK

For this experiment, two strains of the PP type (16 and 70) and two strains of the PK type (8 and 6) were used as parents. All the crosses gave F_1 plants of the LPA type and the F_2 progenies consisted of PA, LPA, PK and PP in a ratio of *ca* 3:6:3:4. This breeding behaviour is in accord with the genotypes assigned previously to the PP (ggHH) and PK (GGhh) parents.

PK × EK

In the case of PK (8, GGhh) × EK (79), the phenotype of the F_1 plants was found to be of the EK type and the F_2 plants segregated into EK and PK types in a 3:1 ratio. It appears therefore that EK is different from PK by another dominant allele.

PP × EK

In the crosses between PP (1 and 16, ggHH) and EK (3 and 79), all the F_1 plants were of the LPA type and the F_2 progenies consisted of PA, LPA, EK and PP types in a 3:6:3:4 ratio. No PK type plant was found among a total of 105 F_2 plants examined. These data cannot be explained by assuming a third gene that would produce elsholtziaketone, but by a series of multiple alleles (G_1, G_2 and g) at the G locus, where G_1 is dominant to G_2, which also is dominant to g. The F_2 data obtained from the crosses, PK × EK and PP × EK, seem to agree with the

theoretically expected values if the genotypes of EK, PK and PP were G_1G_1hh, G_2G_2hh, and ggHH, respectively. This postulation was tested by backcrossing the F_1 hybrid between PP (1) and EK (3) strains to the PP parent (1). The backcross data showed the segregation for 10 PA (G_1gHH), 5 LPA (G_1gHh), and 20 PP (ggH-) plants, supporting the validity of the postulated genotypes.

Taking the existence of the multiple alleles into consideration, all the results presented in Table 2 are fully explained by assigning the following genotypes to the parental strains, G_1G_1HH to strain 76 (PA); G_2G_2HH to 9, 32 and 75 (PA); G_1G_1hh to 3 and 79 (EK); G_2G_2hh to 6, 8 and 11 (PK); ggHH to 1, 16 and 70 (PP) and gghh to 5 and 12 (PP). Table 3 shows the relationship between the phenotypes and their corresponding genotypes proposed on the basis of the present experiments.

DISCUSSION

The present studies have clearly demonstrated that the chemotypes of *P. frutescens* are based on genetic differences and that the chemical compositions of the volatile oils are controlled by two independent genes. The possible roles of these genes may be considered in view of the hypothetical biosynthetic pathways proposed by Hegnauer [4] and modified by us [3] (Scheme 1). In this scheme, four different pathways leading to various metabolites have been postulated in consideration of their chemical structures; (1) geranyl pyrophosphate (GPP) → L-limonene → perillaldehyde, (2) GPP → *cis*-citral → *trans*-citral → elsholtziaketone, (3) GPP → *cis*-citral → perillaketone and isoegomaketone, and (4) shikimic acid → myristicin, dillapiol and elemicin.

It has been found that either one of the multiple alleles, G_1 and G_2, is essential to the synthesis of monoterpenoids in *P. frutescens*. However, the resulting acyclic monoterpenoid metabolites are distinctly different in chemical structures between the two genotypes G_1-hh and G_2-hh, specifically in the positions of both furan ring and ketone. Therefore it is possible that G_1 might control the oxidation of *trans*-citral, whereas G_2 might control that of *cis*-isomer, although this possibility must be confirmed by biochemical studies at the enzyme level. Such genetic control at the parting of the branched biosynthetic pathways has also been reported on the volatile oil components of *Mentha*; carvone (2-oxygenated compound) is synthesized in the presence of the dominent allele C, whereas piperitenone (3-oxygenated) is derived from L-limonene [5] in the recessive genotype cc [6]. As regards the positions of oxidation, alleles G_1 and G_2 of *Perilla* seem to be analogous to alleles C and c of *Mentha*, respectively. The functional difference between G_1 and G_2 is also comparable to that between the multiple alleles in the P locus of *Mentha*, where P^r and P^s control the conversion of (+)-pulegone into (+)-isomenthone and (−)-menthone, respectively [7].

Table 3. Proposed genotypes corresponding to five kinds of phenotypes for the chemical composition of the volatile oil in *Perilla frutescens*

Phenotype	Proposed genotypes
PA	G_1-HH, G_2-HH, G_1G_2HH
LPA	G_1-Hh, G_2-Hh, G_1G_2Hh
EK	G_1hh, G_1G_2hh
PK	G_2-hh
PP	ggHH, gghh

It has been found that all the chemotypes producing monoterpenes fail to produce such specific phenylpropanoids as myristicin, and vice versa. Apparently, the formation of these phenylpropanoids is completely suppressed in the presence of either G_1 or G_2 that exclusively activates the synthesis of monoterpenoids (Scheme 1). It is of interest that these remote biosynthetic systems appear to be controlled alternatively by the same gene, although its biochemical regulatory mechanism is unknown.

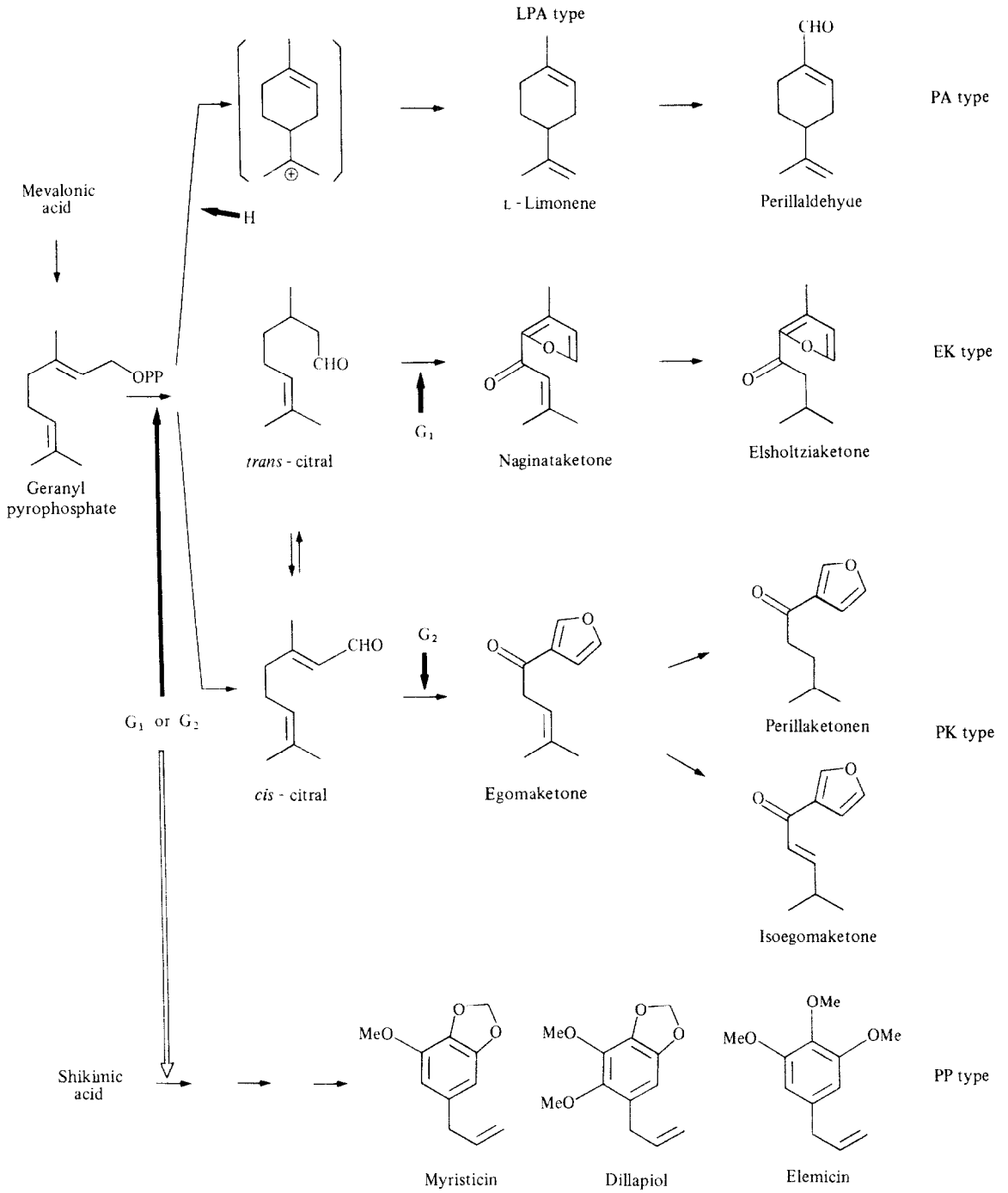
In contrast to such monoterpene ketones as elsholtziaketone and perillaketone which are formed in the presence of only one dominant allele G_1 (or G_2), the synthesis of the monoterpene aldehyde (perillaldehyde) requires the coexistence of G_1 (or G_2) with another dominant allele H. This gene probably controls the cyclization of a precursor such as geranyl pyrophosphate or its direct derivative. Furthermore, the quantitative proportion of perillaldehyde to L-limonene depends on the dose of H, i.e. the former is almost completely replaced by the latter when the plant is heterozygous for H. This gene dosage effect would be expected if L-limonene is an intermediate in the biosynthetic pathway leading to perillaldehyde, the rate of its conversion into perillaldehyde being dependent on the dose of H. In *Mentha*, on the other hand, Murray *et al.* [8] have found the accumulation of L-limonene in the presence of the dominant allele Lm that blocks the conversion of L-limonene into piperitenone.

Although at least four chemical varieties of *Perilla* have been cultivated for commercial or domestic use in Japan, the variety of the PA type is generally considered to be preferable to the others as a spice or crude drug because of the agreeable fragrance of perillaldehyde. In contrast to the PA type, the leaves of both EK and PK types have the disagreeable odour of terpene ketones, while the PP type is odourless. Recently, Wilson *et al.* [9] have reported the occurrence of lung oedema among the cattle grazing *Perilla* plants of the PK type demonstrating that perillaketone is the toxic principle. According to Seto *et al.* [10], myristicin contained in the PP type is hallucinogenic. In view of these findings, the varieties of the PK and PP types would be undesirable for human use.

EXPERIMENTAL

Plant material. Six strains (1, 5, 8, 11, 12 and 76) of the green-leaf variety 'Aojiso' (*P. frutescens* Britton var. *acuta* Kudo f. *viridis* Makino), seven strains (3, 6, 9, 32, 70, 75 and 79) of the red-leaf variety 'Akajiso' (*P. frutescens* Britton var. *acuta* Kudo), and one strain (16) of the variety 'Katamenjiso' (*P. frutescens* Britton far. *acuta* Kudo f. *crispidiscolor* Makino) having leaves with the red, reverse face were intercrossed for genetic analysis. All the strains used as parents for intercrossing had been self-pollinated for more than two generations to confirm that no genetic segregation for chemical compositions of volatile oils occurred in the progeny plants. The method of artificial pollination has been described previously [11].

GC analysis of volatile oils. Fresh leves sampled from individual plants were extracted with Et_2O (1.25 ml/g fr. wt) overnight at 4°. GC analysis was carried out using a stainless column (3 mm × 2 m) packed with PEG-6000 chromosorb W 17% (30–60



Scheme 1. Possible reaction steps controlled by multiple alleles, G_1 and G_2, and another allele H in the hypothetical bisoynthetic pathways of the volatile oil constituents of *Perilla frutescens*. ⬅, Promotion; ⇦, inhibition.

mesh) at a column temp. of 170°, N_2 at 30 ml/min, FID [3, 12–14]. The major compounds of volatile oils were identified by their *RR*, (perillaldehyde = 1) and by comparison with authentic samples [3, 12–14].

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