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Genetics of Monoterpene Biosynthesis in Perilla Plants

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Abstract

This review deals with the biological aspects of monoterpene biosynthesis with special reference to the genetic nature of chemical variations (chemotypes) and a number of genes controlling biosynthetic reaction steps leading to the terpenoid components of essential oils in *Perilla frutescens*. Genetic analyses revealed the existence of two dominant genes of basic importance: gene *G* required for the induction of the whole monoterpene biosynthesis and gene *H* controlling a monoterpene cyclase (limonene synthase) necessary for the formation of cyclic monoterpenes. The nature and biochemical roles of these interesting genes are discussed in detail.

Introduction

Monoterpenes constitute the major portion of the essential oils in the flowers and leaves of plants, but few studies have been made on the genetic control of monoterpene biosynthesis since Murray (1960a,b) investigated the genetic basis of chemical variation in *Mentha* monoterpenes, despite recent advances in the biochemistry (Croteau, 1986) and the ecological chemistry (Bergström, 1991) of plant monoterpenes. To develop the knowledge of genetic control mechanisms of terpene biosynthesis is an essential factor not only in the understanding of chemical diversity in the essential oils but also in the genetic improvement of the scents or flavors of flowers or fruits, the qualities of spices, and the potencies of medicinal plants.

This motivated us to study the genetic basis of chemical variation in the essential oil components of a labiate species, *Perilla frutescens* Britton (Shiso, perilla), which is widely cultivated in Japan and used for flavoring, garnish, and medicine (Tabata, 1997). It is known that the essential oil of *Perilla* is accumulated mainly in the peltate glandular trichomes of the leaf (Nishizawa *et al.*,1992a) and that its agreeable aromatic smell largely depends on the presence of a cyclic monoterpene, perillaldehyde (4-isopropenyl-1-cyclohexene-1-carboxaldehyde), which is an important chemical factor both commercially and medicinally. However, there are chemical varieties (chemotypes) of this species which do not have perillaldehyde but either a monoterpene with a disagreeable smell or a phenylpropanoid as the main oil component.

We chose this annual plant as a material suitable for genetic studies, because four different chemotypes regarding essential oil components had been reported for the local varieties (Ito, 1970) and intercrosses between the self-pollinated strains of different chemotypes could readily be made in order to study the mode of inheritance (Koezuka *et al.*, 1985a).

Chemotypes: variations in chemical components

First we examined the chemical components of essential oils extracted with ethyl ether from the fresh leaves of 215 local strains of *P. frutescens*, which we had collected at random from various places in Japan such as farms, roadsides, and mountains. These materials were cultivated in the same field in Kyoto under much the same conditions in order to make the fair comparison of essential oils possible. Although all the materials contained essential oils with an average content of 0.3% of fresh leaves, the kinds of components varied with local varieties or strains.

Analyses of the leaf extracts by gas chromatography indicated that these strains can be classified into five chemotypes with respect to differences in the major oil components (**Fig. 1**): [1] perillaldehyde (PA) type with an agreeable aromatic odor: 140, [2] perillaketone (PK) type: 12, and [3] elsholtziaketone (EK) type with a disagreeable odor: 31, respectively, [4] a rare citral (C) type with a sweet lemon-like odor: 1, and [5] phenylpropanoid (PP) type with a faint disagreeable odor: 31 (Koezuka *et*

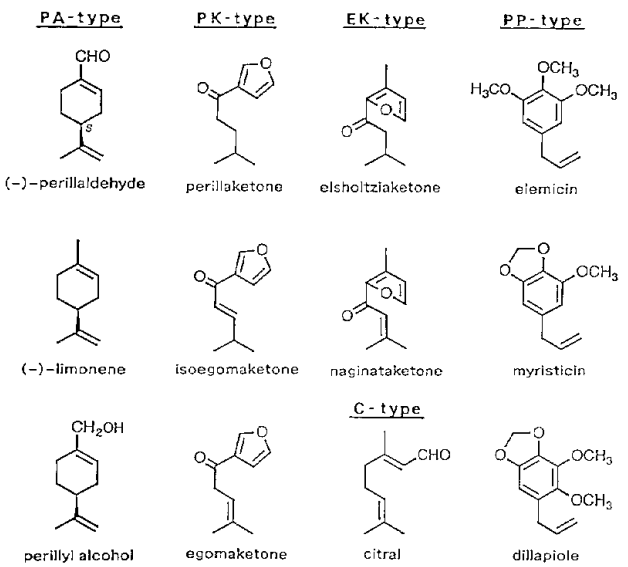

Fig. 1. Major essential oil components of various chemotypes of *Perilla frutescens*.

al., 1984).

The *PA chemotype* contains cyclic monoterpenes consisting of perillaldehyde and smaller amounts of limonene and perillylalcohol. The aromatic compounds perillaldehyde and limonene occupy ca. 70% and 9% of the oil, respectively. This type is preferably used for a spice and a crude drug. By contrast, both *PK and EK chemotypes* contain only acyclic monoterpenes of the furylketone type characterized by a ketone and a furane ring. The furylketones of PK and EK types are distinguished from each other by the positions of oxidation. We have proved that perillaldehyde or perillaketone are the active principles of the perilla leaves responsible for antidermatophytic (Honda *et al.*, 1984), sedative (Honda *et al.*, 1986,1988) or intestinal propulsion activities (Koezuka, 1985b). Wilson *et al.* (1977) reported, however, that perillaketone caused serious lung edema in the cattle grazing wild perilla in the pasture, warning the danger of growing a perilla variety of the PK type as a feed.

The *C type*, a new chemotype which we found by chance contains only an acyclic monoterpene, citral, which is known as the main component of the lemongrass oil.

The *PP type* is of great interest because it contains no monoterpene but one or two of the phenylpropanoids; elemicin, myristicin, and dillapiole, which are not present in the other chemotypes of perilla but in nutmeg. Myristicin is reportedly a hallucinogen (Shulgin, 1966), while dillapiole shows a strong sleep-prolonging activity on mice (Honda *et al.*, 1988).

The results of these studies indicated that the chemotype or the chemical composition of the essential oils not only determines the scents but the pharmacological effects of perilla leaves used for Kanpo or traditional Chinese medicine (Chen, 1997).

Genetic control of monoterpene biosynthesis

Fig. 2 shows a theoretically constructed scheme proposed by Hegnauer (1966) for the biosynthesis of *Perilla* monoterpenes from a common precursor, geranylpyrophosphate (GPP) through branched pathways; perillaldehyde via (-)-limonene, perillaketone via *trans*-citral, and elsholtziaketone via *cis*-citral. To verify this hypothesis, we carried out the following genetic experiments:

Intercrosses were made between the strains of four different chemotypes (PA, PP, PK, EK) to analyze the essential oils of the F_1 hybrids and the F_2 progeny plants. As shown in **Table 1**, the PA type was dominant over all other types. All the F_2 progenies segregated for two to four chemotypes in Mendelian ratios expected from differences in one or a few pairs of alleles. The breeding behaviors in these crosses can be fully explained by assuming three pairs of independent genes (Koezuka *et al.*, 1986a).

On the basis of genetic data, we assigned a genotype to each chemotype (**Table 2**). The dominant gene *G* is considered to be essential for the initiation of monoterpene biosynthesis, while the second dominant gene *H* is required for the formation of such cyclohexene derivatives as limonene and perillaldehyde. In addition, the third dominant gene *N* is thought to be necessary for the formation of an acyclic monoterpene, elsholtziaketone (Yuba *et al.*, 1995).

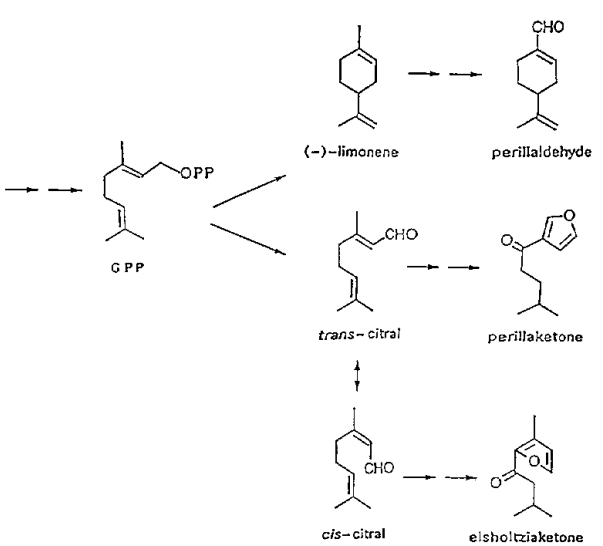

Fig. 2. A theoretical scheme for monoterpene biosynthesis in perilla proposed by Hegnauer (1966).

Table 1. Genetic segregation in the F_2 progenies of the intercrosses between *Perilla* strains showing different chemotypes

Cross	Phenotype of F_1	Number of F_2	Genetic segregation in F_2	
			Phenotype	Ratio
PA × PP	PA	52	PA:PP	3:1
PA × PP	LPA	81	PA:LPA:PK:PP	3:6:3:4
PA × PK	LPA	108	PA:LPA:PK	1:2:1
PA × EK	LPA	51	PA:LPA:EK	1:2:1
PK × PP	LPA	95	PA:LPA:PK:PP	3:6:3:4
EK × PP	LPA	135	PA:LPA:EK:PP	3:6:3:4
PK × EK	EK	42	EK:PK	3:1

PA : perillaldehyde, PK : perillaketone, EK : elsholtziaketone,
PP : phenylpropanoid, LPA : limonene (++) + perillaldehyde (+) .

Table 2. Proposed genotypes for various chemotypes of perilla

Chemotype	Proposed Genotypes
PA	*GG HH NN, GG HH nn*
EK	*GG hh NN*
PK	*GG hh nn*
PP	*gg HH NN, gg HH nn, gg hh nn*

LPA type (hybrid) : *GG Hh* or *Gg Hh*.

The possible roles of these three genes are allotted to the corresponding reaction steps on biosynthetic pathways (**Fig. 2**). It is assumed that gene *G* is involved with the regulation of the biosynthesis leading to GPP, the central precursor of all the monoterpenes. Gene *H* then would work on the cyclization of GPP to give limonene. Thus under the co-existence of two dominant genes, *G* and *H*, cyclohexanoid monoterpenes will be produced. This is the first demonstration of a remarkable metabolic system in which a single dominant gene controls the whole monoterpene biosynthesis. On the other hand, gene *N* is needed for an oxidation step leading to an acyclic monoterpene, elsholtziaketone, from *cis*-citral in the EK chemotype that lacks the *H* gene.

Other genes controlling monoterpene biosynthesis

Further genetic experiments using different strains of perilla have revealed the existence of at least seven other genes (*P, Q, R, J, I,* Fr_1 and Fr_2) which are involved in biosynthetic steps leading to various monoterpenes (**Fig. 3**).

Analyses of acyclic monoterpene biosynthesis was facilitated by finding two natural subgroups of the EK chemotype, which showed significant differences in the proportion of naginataketone to elsholtziaketone. Intercrosses between them suggested that a couple of polymeric genes, *P* and *Q*, promote additively the reduction of naginataketone into elsholtziaketone (Nishizawa *et al.*, 1991). Without these genes, the reaction was completely blocked to accumulate only naginaketone but no elsholtziaketone.

We were also fortunate to find in a mountain a wild perilla plant whose essential oil consisted mainly of perillene (96%) and a small amount of citral (4%). The F_2 and backross data obtained from crosses between the PL (perillene) chemotype and the PK or EK chemotype showed that the PL type lacked a dominant gene, *J*, which is required for the oxidation of perillene at the C-6 position to yield egomaketone (Nishizawa *et al.*, 1990a).

Furthermore, we have demonstrated that egomaketone is reduced to a final product perillaketone in the presence of a dominant inhibitor gene, *I*, by both genetic and tracer experiments (Koezuka *et al.*, 1986c; Nishizawa *et al.*, 1989). The gene *I* blocks the isomerization of egomaketone to form isoegomaketone. Without this gene, perillaketone and isoegomaketone are produced in nearly equal amounts.

The conversion of *trans*-citral into perillene through furan formation proved to be controlled by a couple of polymeric genes, Fr_1 and Fr_2, by crosses using a variant strain of the C-type that accumulates *trans*-citral as a major oil component (Yuba *et al.*, 1995). The C type incapable of metabolizing either *trans*- or *cis*- citral was found to be homozygous recessive for those polymeric genes as well as for the gene *N*.

The use of a C type strain for cross experiments provided us with valuable information for deter-

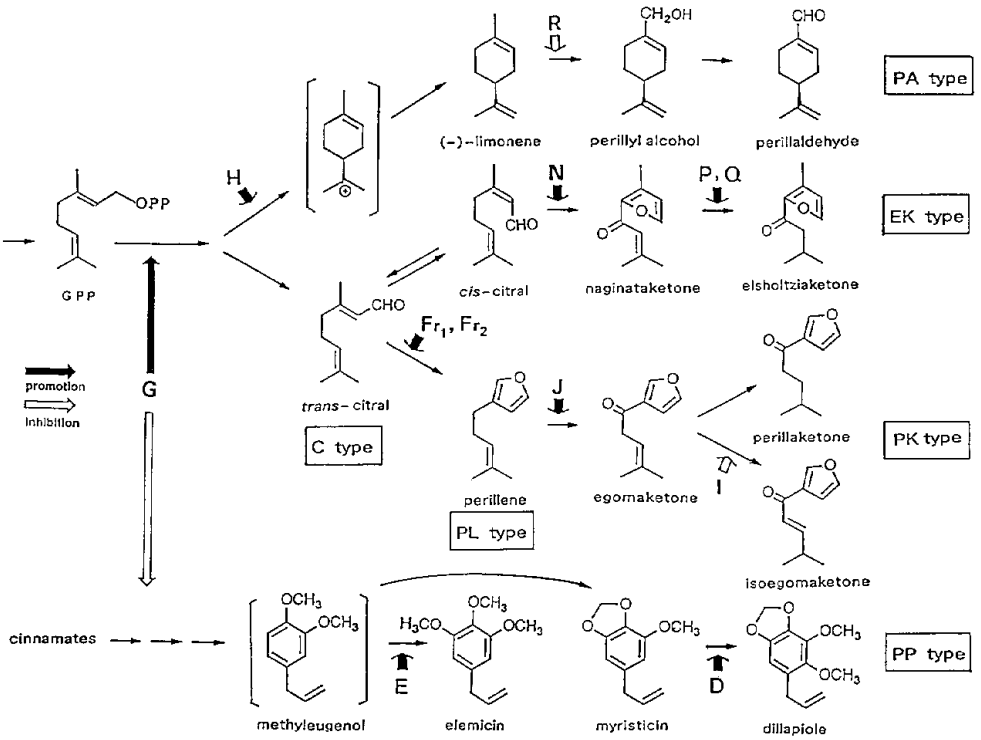

Fig. 3. Proposed biosynthetic pathways for essential oil components and genes controlling the reaction steps in *Perilla frutescens* (Yuba *et al.*, 1995).

mining the genotypes of various chemotypes. F_1 hybrids between the C type and five other chemotypes gave segregation ratios expected from differences in one to four pairs of genes in the F_2 generation. On the basis of these results, the genotypes corresponding to six phenotypes including the C type have been proposed (Yuba *et al.*, 1995) (**Table 3**).

The dominant gene *G* is present in all the chemotypes producing monoterpenes, while the dominant gene *H* is present not only in the PA chemotype but occasionally in PP type strains producing no monoterpenes. The *H* gene is completely epistatic to the genes *Fr* and *N*, which are involved in the biosynthesis of acyclic monoterpenes. A dominant gene designated as *R* was found only in the EK and PK type strains. The *R* gene is closely linked with the recessive allele *h*, partially inhibiting the oxidative conversion of limonene into perillylalcohol and perillaldehyde.

Genetic control of phenylpropanoid biosynthesis

In the absence of gene *G*, perilla plants accumulate unusual phenylpropanoids in place of monoterpenes in the essential oils. These compounds are characterized by having an allyl group but are different from each other in the substitution pattern of the benzene ring. We classified the strains

Table 3. Proposed genotypes based on crosses between the C type and various chemotypes of *Perilla*

Phenotype	Genotypes
PA (perillaldehyde)	*GG HH rr nn FF ff jj*
	GG HH rr nn ff ff jj
EK (elsholtziaketone)	*GG hh RR NN ff ff jj*
PK (perillaketone)	*GG hh RR nn FF FF JJ*
PL (perillene)	*GG hh rr nn FF ff jj*
C (citral)	*GG hh rr nn ff ff jj*
PP (phenylpropanoids)	*gg HH rr nn FF ff jj*
	gg hh RR nn FF FF JJ

H and R are closely linked.
Two sets of FF are independent polymeric genes.
N is epistatic for F.

of the PP type into three subgroups: (1) M type containing only myristicin, (2) DM type containing dillapiole and a small amount of myristicin, and (3) EM type containing elemicin and a smaller amount of myristicin.

The genotypes of these variant strains with respect to phenylallyl derivatives were determined by the F_1 and F_2 data obtained from intercrosses to be *ddee*, *DDee*, and *ddEE* for M, DM, and EM types, respectively (Koezuka *et al.*, 1986b). As shown in **Fig. 3**, a possible precursor, methyleugenol, would be metabolized to elemicin in the presence of a dominant gene *E*. In the absence of this gene,

however, myristicin will be produced by the formation of a methylenedioxy group. Then myristicin might be metabolized further into a final product, dillapiole, through hydroxylation and methylation in the benzene ring, if the plant carried a dominant gene *D*.

It is an open question whether a single gene *G* that activates monoterpene biosynthesis simultaneously inhibits the metabolism of cinnamates to phenylallyl derivatives by a pleiotropic effect on two separate biosynthetic pathways. This interesting but puzzling problem, and will be discussed later on from the biochemical standpoint.

Genic control of a monoterpene cyclase

A great number of enzymes catalyzing reaction steps involved in the biosynthesis of perilla monoterpenes have not been elucidated biochemically, but our effort has been focused on the relationship between the *H* gene and limonene synthase, which is a monoterpene cyclase catalyzing the cyclization of GPP to yield limonene, an intermediate leading to perillaldehyde.

Kjonaas and Croteau (1983) demonstrated that limonene is the first cyclic intermediate derived from GPP in the biosynthesis of oxygenated monoterpenes in the peppermint. **Fig. 4** illustrates a scheme proposed by Croteau as the mechanism of the cyclization of GPP, which involves the initial ionization of the pyrophosphate moiety with the assistance of a divalent cation, followed by rotation about the C-2 - C-3 bond and subsequent cyclization (Rajaonarivony *et al.*, 1992). Since a similar cyclization process is expected of perilla, we assayed limonene synthase activity in relation to gene *H* (Nishizawa *et al.*, 1992b).

Tracer experiments with cell-free extracts prepared from 1-week-old cotyledons of different chemotypes showed that ^{14}C-labeled GPP was efficiently converted to limonene only in the PA chemotype having both dominant genes *G* and *H*. The enzymatic reaction showed absolute requirement for manganese ion which could not be replaced by any other divalent metals. By contrast,

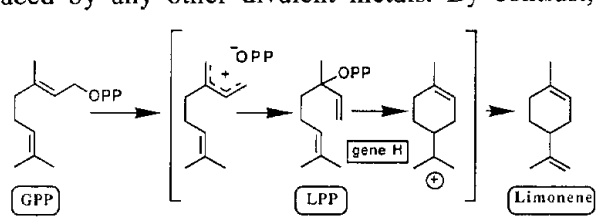

Fig. 4. Hypothetical gene-controlled reaction steps involved in the cyclization of geranylpyrophosphate (GPP) via linallyl pyrophosphate (LPP) (Nishizawa *et al.*, 1992b).

labeled GPP was never incorporated into limonene in any plant without a dominant gene *G*, even if it carried gene *H*. It suggested that gene *H* cannot be expressed in the absence of gene *G*. The results clearly indicate that the dominant gene *H* controls the key enzyme limonene synthase.

Characterization of limonene synthase cDNA

For a molecular biological study on perilla limonene synthase, we constructed a DNA library from cotyledons of the PA chemotype homozygous for both dominant genes *G* and *H*; then we isolated cDNAs homologous to the spearmint limonene synthase cDNA, which was a generous gift from Prof. Croteau of Washington State University.

The sequence analysis of a cDNA clone containing the longest open reading frame showed that it consisted of 1812 nucleotides corresponding to 603 amino acids (Yuba *et al.*, 1996). The identity of this amino acid sequence in comparison with that of spearmint limonene synthase (Colby *et al.*, 1993) was as high as 65%. The sequence ranging from the 350th to 356th amino acid in the spearmint cyclase, which was presumed to be a metal-substrate complex binding site, is well conserved by perilla.

Expression of gene *H* for limonene synthase

Genomic Southern blot analyses of 20 perilla strains of various chemotypes were carried out by using a 3'-flanking region of the cDNA encoding limonene synthase as a probe. The analyses revealed the presence of two bands in both PA and PP strains which were homozygous for the dominant allele *H*. In contrast, no such DNA sequences were found in other strains lacking gene *H* (**Table 4**).

Northern blot analyses using the 3'-flanking

Table 4. Genomic Southern analysis of 20 perilla strains of the various genotypes. Total DNA isolated from leaves was digested with either *Hind* III or *Eco* RI, followed by electrophoresis, blotting, and hybridization with a DNA fragment from the 3'-flanking region of pPFLC1, as a sequence-specific probe.

Chemotype	Number of strains	Genotype	Southern blot analysis
PA	3	*GG HH*	two bands
PK	7	*GG hh*	no band
EK	4	*GG hh*	no band
PL	1	*GG hh*	no band
PP	4	*gg HH*	two bands
PP	1	*gg hh*	no band

region of the same cDNA as a hybridization probe also showed that the corresponding mRNA accumulated in all the aerial parts, particularly in the leaves of plants homozygous for both dominant genes *G* and *H*. By contrast, such mRNA was hardly detectable in any plants lacking *G*. This observation is in accord with the results of the enzyme assay for cotyledons that no limonene synthase activity was detected in the absence of *G*, even if the plant carried *H*. The reason why gene *H* can be expressed only in the presence of gene *G* is not clear. However, if *G* should be responsible for a transcription factor having an affinity in common with the promoter region of *H*, the expression of *H* would depend on the presence of gene *G*.

The cDNA encoding perilla limonene synthase was functionally expressed in *Escherichia coli*, yielding an enzyme capable of converting the substrate GPP into limonene, which was confirmed by GC-MS analysis. Therefore, it is considered that the dominant gene *H* must be either the limonene synthase structural gene itself or a gene locus containing a DNA sequence encoding this enzyme as a part of its structure.

A possible functional role of the *G* gene

What might be the intriguing functional or biochemical role of the master gene *G* vital to the biosynthesis of both cyclic and acyclic monoterpenes? The possibility that gene *G* might control GPP synthase (Croteau and Purkett, 1989) for the synthesis of GPP from the two building blocks, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), was rejected by the examination of GPP synthase activity. For this assay, ^{14}C-labeled IPP and cold DMAPP were incubated with a crude enzyme extracted from the cotyledons of each chemotype, and the enzymatic reaction products were hydrolyzed with acid phophatase to liberate geraniol and a sesquiterpene, farnesol. The results showed that IPP was incorporated into geraniol as well as farnesol irrespective of the genotype, indicating that GPP synthase was equally active in all of the genotypes, whether or not cotyledons carried the gene *G* (Nishizawa *et al.*, 1992b). It is therefore suggested that gene *G* controls a reaction step prior to the formation of IPP.

Another possibility regarding the role of the gene *G* is that it might be involved in the regulation of a non-mevalonate pathway leading to IPP and monoterpenoids, which has recently been demonstrated for higher plants by Eisenreich *et al.* (1997, 1998). In this alternative pathway, IPP is not derived from mevalonic acid but from a key precursor, 1-deoxy-xylulose 5-phosphate (DXP). These findings seem to explain the results of our experiments that neither ^{14}C-labeled acetate nor mevalonate but only sucrose was incorporated into monoterpenes in perilla. The ^{14}C-labeled mevalonate was incorporated into a sesquiterpene, caryophyllene, which is probably derived from the classical mevalonate pathway and accumulates in a significant amount in all the chemotypes of perilla (Nishizawa *et al.*, 1990b).

Eisenreich *et al.* (1998) proposed that the key intermediate DXP is synthesized by DXP synthase catalyzing the condensation of glyceraldehyde 3-phosphate with pyruvic acid bound to thiamine pyrophosphate. This enzyme probably plays a key role in the biosynthesis of plastid-derived isoprenoids in the peppermint (Lange *et al.*,1998). Thus it seems likely that the *G* gene of perilla might be controlling this key enzyme, although there is no direct evidence to support this hypothesis (**Fig. 5**).

In the absence of gene *G*, it may be speculated that glyceraldehyde 3-phosphate, which can not be used for the synthesis of DXP because of a genetic block, might be converted to erythrose 4-phosphate and used for the synthesis of shikimic acid. In that case, an excess flow of cinnamates from the shikimate pathway might be metabolized to form unusual phenylpropanoids, such as myristicin and elemicin. Presumably, the alternative production of monoterpenes or phenylpropanoids may be due to preferential distribution of such a precursor as glyceraldehyde 3-phosphate located at a junction where two biosynthetic tracks branch off.

Localization of monoterpene biosynthesis

In *Thymus vulgaris* (thyme), the biosynthesis and accumulation of the major monoterpenoid component thymol proved to take place exclusively in the intact glandular trichomes isolated from the cotyledons (Yamaura *et al.*,1992). Colby *et al.* (1993) reported that the limonene synthase activity resides at the glandular trichomes in spearmint. In perilla, the amount of oil components accumulated in the leaf was positively correlated with the number of peltate glandular trichomes (PGT), suggesting PGT is the main site of monoterpene biosynthesis (Nishizawa *et al.*, 1992a). The electron microscopy of secretory cells in PGT on 1-week-old perilla cotyledons that have begun producing the essential oil by Prof. Matsushima of Saitama University (personal communication) revealed increasing numbers of endoplasmic reticulum, Golgi bodies and plastids showing dark deposition of the high electron density. In this connection, Gleizes *et al.*

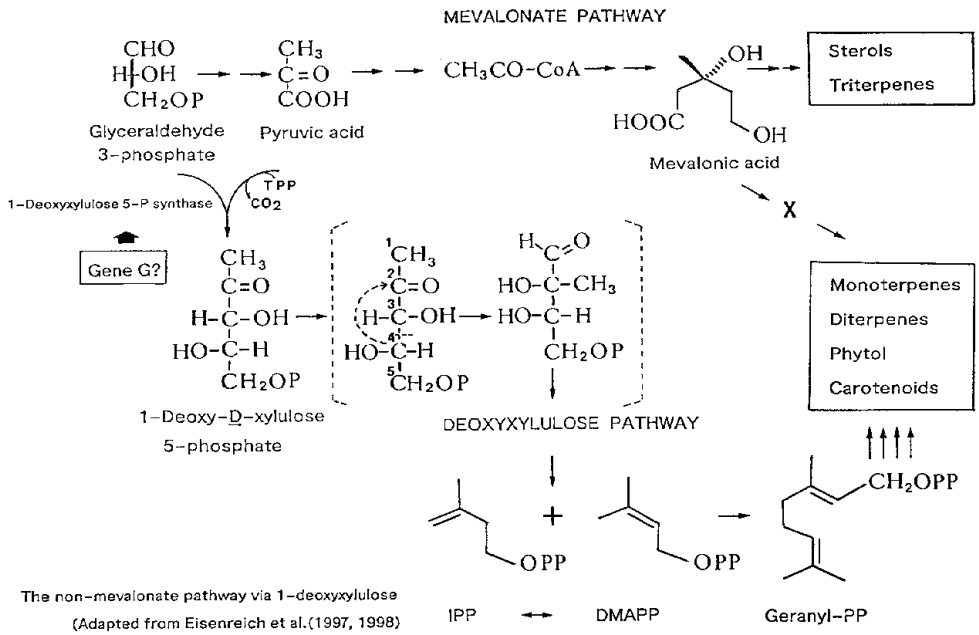

Fig. 5. A hypothetical scheme for a genetic control of the non-mevalonate pathway leading to the formation of terpenoids in *Perilla frutescens*.

(1983) reported the formation of limonene from IPP supplied to a leucoplast preparation from the excarp of calamondin fruits, suggesting that the plastid is the site of monoterpene biosynthesis. It seems likely that the monoterpene biosyntehsis in perilla also takes place in plastids under the control of nuclear genes.

Conclusion

The present study has clarified the genetic basis of chemical variation with respcet to essential oil components in perilla. A new finding of great interest is that the initiation of the whole monoterpene biosynthesis is controlled by a master gene, *G*. It is also important that the enzymatic formation of cyclic monoterpenes depends on another dominant gene, *H*, which functions only in the presence of *G* gene. Perhaps there is a possibilty that a similar genetic control system might be operating on the determination of basic chemical types of essential oils in some other plant species as well. For example, such "chemical races" with respect to the monoterpenoid components of essential oils as were reported for *Eucalyptus* and *Pinus* (Alston and Turner, 1963; Smith, 1976) might be well explained by postulating two pairs of alleles similar to *G/g* and *H/h* found in perilla. On the other hand, these genes may be utilized biotechnologically for breeding transgenic plants with an altered terpenoid composition.

Although our knowledge of the genetic control of monoterpene biosynthesis has been extended. a number of important problems including the regulatory mechanisms of gene expression in the secretory cells of glandular trichomes remain to be investigated. The genotypically defined perilla chemotypes would provide useful materials for future studies. From our experiences in the present work on chemical diversity, we cannot too greatly emphasize the importance of doing more extensive *biological* studies including "classic" genetics, biochemistry and physiology as well as molecular biology for a better understanding of plant secondary metabolism.

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