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# 들깨 Limonene 유전자의 담배식물(*Nicotiana benthamiana*)내 Agroinfiltration에 의한 분자적 특성

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# Molecular Characterization of the *Perilla frutescens* Limonene Gene (PFLS) by Agroinfiltration into *Nicotiana benthamiana*

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**ABSTRACT : The full-length cDNA encoding *Perilla frutescens* limonene synthase (PFLS) (603 amino acids, GenBank accession no. D49368) was cloned. To elucidate the role of PFLS in gene regulation, we transiently transformed full-length *PFLS* into tobacco plants. *PFLS* mRNA was first detected in the intact leaves of the plants at 6 h, and the *LS* transcript level increased after 12 h in leaves treated with oxidative stress-related chemicals. The transient overexpression of PFLS resulted in increased transcription of *NbPR1* and *NbSIP* in *Nicotiana benthamiana* leaves. Thus, our result confirmed that the infiltration of PFLS gene act as a transcriptional regulator of NbPR1 or NbSIP genes in the tobacco.**

***Key Words*** **:** NbPR1, NbSIP1, Oxidative Stresses, PFLS, Transient Overexpression

## INTRODUCTION

*Perilla frutescens* (Labiatae) and its wild relatives are widely grown in Japan, China, Korea, Vietnam, and East Asia and used as a source of food, medicine, natural pigment, spice, or oil (Ito and Honda, 1996). The specific odor components of the leaves are related to the essential oil composition and have been detected by gas chromatography-mass spectrometry (GC-MS), (Ito *et al.*, 1999). Limonene synthase was cloned to investigate its function in the biosynthesis of perillaldehyde (PA) produced mainly in specific types of this plant (Yuba *et al.*, 1996). The chemotype generally used as a food or in medicine is the PA (perillaldehyde with cyclohexanoid monoterpenes) type, whereas the PK (perillaketone with furanoid monoterpenes), EK (elsholztiaketone), and PP (phenylpropanoid with phenylpropanoids) types are used as foods (Ito *et al.*, 2002). Furanoid monoterpenoid (+)-menthofuran synthase was cloned by Bertea *et al.* (2001) and is a cytochrome P450-relevant synthase that forms a furan ring *via* an intermediate from geranyl diphosphate (GPP) (Bertea *et al.*, 2001).

Genetic transformation, a plant transient expression technique, has been used to infiltrate plant leaves in liquid culture. This method is useful to study aspects of plant molecular biology, including the specificity of plant promoters and transcription factors (Liu *et al.*, 2003), gene-for-gene interactions between host resistance, pathogen avirulence

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genes (Scofield *et al.*, 1996), foreign gene expression (Kapila *et al.*, 1997), gene silencing (Voinnet *et al.*, 2003), the hypersensitive response (Palanichelvam *et al.*, 2000), and transient expression of antibodies (Vaquero *et al.*, 1999).

We cloned a limonene synthase (LS) gene that is related to the biosynthesis of essential oils in a *P. frutescens* strain that has been preserved by our research group (NCBI accession number D49368). In this study, we applied *Agrobacterium*-mediated transient expression to express the LS gene in *Nicotiana benthamiana* plants.

## MATERIALS AND METHODS

### 1. Isolation and vector construction of LS gene from *Perilla frutescens*

A full-length cDNA of the *P. frutescens* LS (PFLS) gene (GenBank No. D49368) was cloned in forward orientation into the pMBP1 vector, yielding pMBP1 : PFLS. For agroinfltration, pMBP1 and the recombinant plasmid (pMBP1 : PFLS) were transformed into *Agrobacterium tumefaciens* LBA4404 via the freeze-thaw method (An *et al.*, 1987). *Nicotiana benthamiana* seeds were germinated and grown in soil in a growth chamber at 25℃ under illumination at 45 lmol m-2 s-1 with cool white fluorescent lights with a 16-h light and 8-h dark photoperiod. A 10-㎖ culture of *Agrobacterium* was grown overnight at 30℃ in YEP medium with 50 ㎎/㎖ kanamycin and 50 ㎎/㎖ rifampicin. The cells were collected by centrifugation (4000 × *g*, 20 min, 20℃), resuspended in infiltration liquid medium (MS salts, pH 5.2), and then exposed to 100 μM acetosyringone at 22-25℃. The *Agrobacterium* mixtures were infiltrated with a needle-less 1-ml syringe into the 5$^{th}$ leaf of 3-week-old *N. benthamiana*. These agro-infiltrated tobacco plants were then grown in a growth chamber at 25℃ with a 16-h light and 8-h dark photoperiod.

### 2. Southern blot analysis

Genomic DNA was prepared according to Dellaporta et al. (1983). Ten micrograms of total DNA were digested with *Hin*dIII or *Eco*RI, and the digested DNAs were separated by size on 0.8% (w/v) agarose gels. Southern blot transfer was carried out using a standard method (Sambrook *et al.*, 1989), and hybridization and washes were performed according to Church and Gilbert (1983). Membranes were hybridized with a $^{32}$P-labeled fragment of the PCR product of the full-length PFLS cDNA in a buffer consisting of 1% BSA, 1 mM EDTA, 0.5 M $NaHPO_4$, pH 7.2, 7% SDS at 65℃ overnight and washed in 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 5% SDS at room temperature for 5 min. The blots were then washed three times with high stringency wash buffer (1 mM EDTA, 40 mM $NaHPO_4$, pH 7.2, 5% SDS) at 65℃. Dried blots were placed on X-ray film at 80℃ for 1 week until fully developed.

### 3. Oxidative stress treatment and Northern blot analysis

For oxidative stress treatments, *P. frutescens* plants were placed on a solution of 50 μM methyl viologen or 10 mM $H_2O_2$ for 24 h. Total RNA was isolated from the inoculated leaf tissues according to a protocol described earlier (Yi *et al.*, 2004). Ten micrograms of total RNA from each sample were fractionated by gel electrophoresis on formaldehyde-containing agarose gels and transferred to a nylon membrane (Amersham, USA). cDNA clones were labeled with $^{32}$P-dCTP and used for Northern blot hybridization.

### 4. RT-PCR for expression analysis

Agro-infiltrated *N. benthamiana* cDNAs were prepared using 1 ㎍ total RNA, and 200 U of SuperScript II (Invitrogen, USA). The perilla actin gene was amplified as the internal equal-loading control using a suitable primer set (forward, 5'-CAGCTCATCCGTGGAGAAGA-3'; reverse, 5'-AGGATACGGGGAGCTAATGC-3'). All other gene-specific primers used in this experiment were designed based on sequences in a previous report (Senthil-Kumar *et al.*, 2007). The upregulated genes and the primer sets for RT-PCR were NbSIP, salt-inducible protein (DQ275463, 5'-TGAGTCAGGCAGGAAGCAAG-3' and 5'-TTTGTACAAG AAAGGTGG-3'), NbPR-1a (5'-ATGGGATTTGTTCTCTTTT C-3' and 5'-TTAGTATGGACTTTCGCCTC3'), and the downregulated gene was NbHSR203J (5'-TGTGTCAGCCA TGCTGATTG-3' and 5'-CCGATAGGACCGCACGAAAC-3') (Kato *et al.*, 2008). The PCR profile was 5 min at 94℃, followed by 21 cycles of 94℃ for 1 min, 55℃ for 1 min, 72℃ for 1 min, and a final extension for 7 min at 72℃. The amplified products were separated on 1% agarose gels and visualized by ethidium bromide staining.

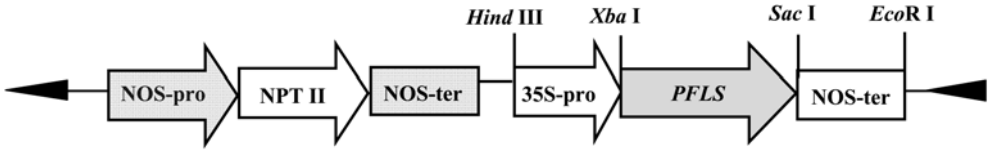


**Fig. 1.** Construction of the plant expression vector. Diagram of the *Perilla frutescens* limonene (PFLS) gene inserted into the plant gene expression vector pMBP1.

## RESULTS AND DISCUSSION

Cloned full-length LS cDNA (D49386) from *P. frutescens* (PFLS) consists of 1812 bp, corresponding to 603 amino acids. The vector pMBP1 contained a single T-DNA harboring the *NPT* II gene driven by the 35S promoter (Fig. 1). PFLS cDNA was amplified by PCR with primers containing restriction sites (*Xba*I/*Sac*I). The PCR product was then amplified, and pMBP1 was digested with same restriction enzymes. The digested vector and PFLS gene were cloned, and the sequence of the ligated PFLS gene was analyzed.

We confirmed a perfect match; the recombinant plasmid was transformed into *Agrobacterium* strain LBA4404 and agro-infiltrated into *N. benthamiana*. Induced terpenoids are important in plant defense mechanisms against herbivores and pathogens. Yuba et al. (1996) cloned the *Perilla* cDNA encoding LS, and nine terpene synthase (TPS) cDNAs were isolated and functionally characterized from Norway spruce (Yuba *et al.*, 1996). *Agrobacterium*-mediated transient assays for the analysis of gene function are used as an alternative to genetic complementation and stable plant transformation. Agroinfiltration has been used to transiently express cre recombinase in planta, and molecular analysis indicated that the recombined state was transferred to the $T_1$ generation, thus demonstrating the suitability of agroinfiltration for in vivo transformation (Lilya and Schiemann, 2005).

The total DNA from perilla was digested with *Hind*III and *Eco*RI, then subjected to Southern blot analysis. The membrane was hybridized with a DNA fragment from the full-length PFLS (Fig. 2A) as a sequence-specific probe. Two bands were observed in DNA digested with either *Eco*RI or *Hind*III, suggesting that there are at least two copies of LS (Fig. 2A). The gene for PFLC1 encoding LS was only detected in strains of the *HH P. frutescens* genotype capable of producing cyclohexanoid-type monoterpenes (Yuba *et al.*, 1996). The dominant *H* gene is

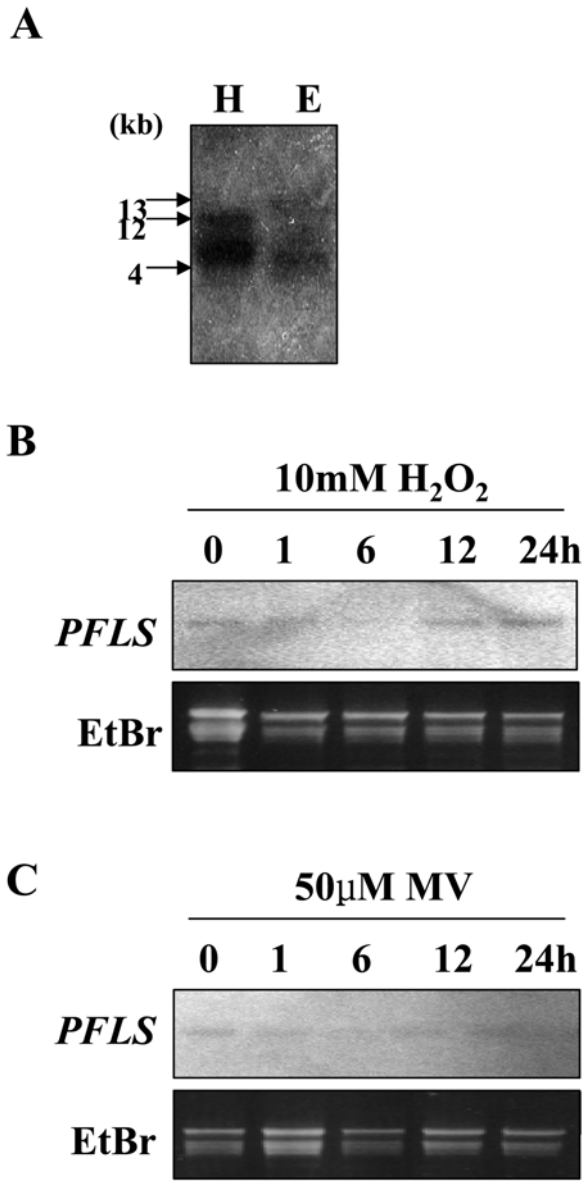


**Fig. 2.** Southern blot analysis of genomic DNA and expression patterns of PFLS during oxidative stresses. **A.** Genomic DNA was digested with *Hind*III (H) and *Eco*RI (E), fractionated on 0.8% agarose gels, and transferred to nylon membranes. The membranes were hybridized with a $^{32}$P-labeled fragment of PCR product containing the full length PFLS cDNA (D49368). **B** and **C.** Responses for $H_2O_2$ and Methyl viologen. Ten microgram samples of total RNA were fractionated on 1% denaturing agarose gels. See Materials and Methods.

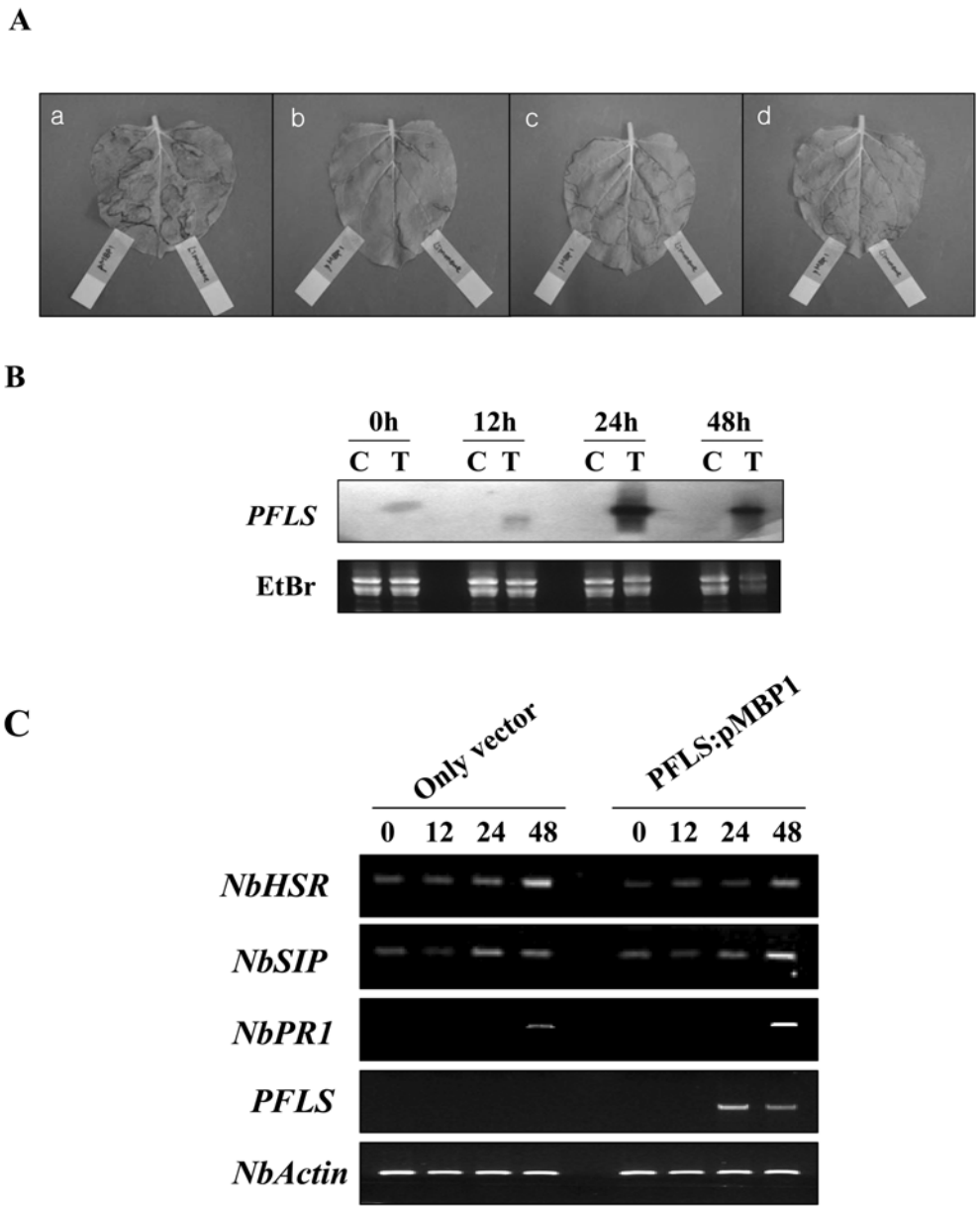


**Fig. 3.** Transient overexpression of PFLS gene in leaves of *Nicotiana benthamiana*. **A.** Tobacco leaves that infiltrated vector only or overexpressed PFLS by *Agrobacterium*-mediated transient transformation (0h, 12h, 24 h, and 48 h). **B.** RNA expression of PFLS gene in Agro-infiltreated tobacco leaves. **C.** Expressions of genes related to biotic and abiotic stresses activated by transient PFLS overexpression in tobacco leaves. The expressions of two defense-related genes, NbHSR203J and NbPR1a, and salt stress-related genes, NbSIP (salt induced protein), were compared by RT-PCR. NbActin was used as a positive control. We used the primers mentioned in Kato et al. 2008. The experiment was repeated at least three times and a representative result is shown.

completely epistatic compared to other genes such as Fr1, Fr2, and N, which are involved in the biosynthesis of non-cyclohexanoid-type monoterpenes (Yuba *et al.*, 1995).

The mRNA levels corresponding to PFLS transcripts in tissues treated with methyl viologen and $H_2O_2$ were investigated by Northern analysis. The RNA blot was probed with the same cDNA fragment as used for genomic Southern analysis. As shown in Fig. 2B and 2C, LS mRNA was first detected in intact leaves of the plants at 6 h, and the LS transcript increased after 12 h in leaves treated with chemicals. Tissue-specific expression analysis demonstrated that PFLC mRNA accumulates in the aerial parts of GGHH plants, with the highest mRNA level in leaves (Yuba *et al.*, 1996). Isoprene-emitting

transgenic lines represent important material for future analyses to understand the role of isoprene in mediating thermotolerance and in protection against oxidative stress (Lucker *et al.*, 2004a; Lucker *et al.*, 2004b). Germin-like proteins (GLPs) are involved in pathogen resistance, but their direct involvement is poorly understood (Lou and Baldwin, 2006). Silencing of NaGLP does not influence OS-elicited jasmonate and salicylate bursts, or the release of volatile organic compounds such as limonene, cis-a-bergamotene, and germacrene-A that function in indirect defense (Lou and Baldwin, 2006).

The recombinant plasmid (pMBP1 : PFLS) was transformed into *Agrobacterium tumefaciens* LBA4404 and then was infiltrated into intact leaves of *N. benthamiana*. (Fig. 3A and 3B). To examine whether PFLS contributes to the expression of genes related to defense and stress, including the PR-1 gene, *Agrobacterium* harboring pMBP1 binary vectors with a coding region for PFLS under control of the CaMV 35S promoter, or vector only, was infiltrated into *N. benthamiana*. After 0, 0.5, 1, and 2 days, total RNA from the infiltrated tobacco leaves was isolated for RT-PCR analysis using NbPR1-, NbSIP-, and NbHSR-specific primers (Fig. 3C). When PFLS was overexpressed in tobacco leaves, NbPR1 and NbSIP genes transcripts increased compared to non-infiltrated and vector control plants, while the transcript level of NbHSR was reduced in the same samples. LS catalyzes GPP to form a monocyclic monoterpene, limonene. In an attempt to engineer monoterpene biosynthesis, three expression constructs of LS cDNA from *P. frutescens* were introduced into tobacco (Ohara *et al.*, 2003). The amount of limonene in LS transgenic plants increased 2.7- to 3.0-fold (Ohara *et al.*, 2003). The availability of constitutive and inducible monoterpene synthases allow molecular dissection of the resin-based defense response in Norway spruce (Martin *et al.*, 2004). The oxygenation pattern of the essential oil monoterpenes of commercial mint (*Mentha*) species was determined by regiospecific cytochrome P450-catalyzed hydroxylation of the common olefinic precursor, (2)-4*S* limonene (Haudenschild *et al.*, 2000). The evolution of terpene synthases in multi-gene families and their differential expression is mediated by plant-environment interactions (Tholl, 2006). Transgenic tobacco plants produced high levels of terpenes by overexpression of the appropriate terpene synthase (Wu *et al.*, 2006). We believe that infiltration-associated PFLS overexpression with *Agrobacterium* probably contributed to upregulation of the NbPR-1 gene.

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