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Molecular Modification of *Perilla* Lipid Composition

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Abstract

In order to modify lipid production of *Perilla* qualitatively as well as quantitatively by genetic engineering, genes involved in carbon metabolism were isolated and characterized. These include acyl-ACP thioesterases from *Perilla frutescens* and *Iris* sp., four different *β*-ketoacyl-ACP synthases from *Perilla frutescens*, and two △15 acyl-ACP desaturases(*Pffad7, Pffad3*). *Δ*15 acyl-ACP desaturase (*Δ*15-DES) is responsible for the conversion of linoleic acid (18:2) to *α*-linolenic acid (ALA, 18:3). *Pffad 3* encodes *Δ*15 acyl- desaturase which is localized in ER membrane. On the other hand, *Pffad7* encodes a 50 kD plastid protein (438 residues), which showed highest sequence similarity to *Sesamum indicum fad7* protein. Northern blot analysis revealed that the *Pffad7* is highly expressed in leaves but not in roots and seeds. And *Pffad3* is expressed throughout the seed developmental stage except very early and fully mature stage. We constructed *Pffad7* gene under 35S promoter and *Pffad3* gene under seed specific vicillin promoter. Using *Pffad7* construct, *Perilla*, an oil seed crop in Korea, was transformed by *Agrobacterium* leaf disc method. *α*-linolenic acid contents increased in leaves but decreased in seeds of transgenic *Perilla*. Currently, we are transforming *Perilla* with *Pffad3* construct to change *Perilla* seed oil composition. We isolated three ADP-glucose pyrophosphorylase (AGP) genes from *Perilla* immature seed specific cDNA library. Nucleotide sequence analysis showed that two of three AGP (*Psagp1, Psagp2*) genes encode AGP small subunit polypeptides and the remaining (*Plagp*) encodes an AGP large subunit. PSAGPs, AGP small subunit peptide, form active heterotetramers with potato AGP large subunit in *E. coli* expressing plant AGP genes.

Introduction

Perilla frutescence is an oil seed crop widely grown in Korea. Vegetable oils from *Perilla* seeds have high nutritional value. Most of fatty acids in the oil are unsaturated and *α*-linolenic acid content of the oil is exceptionally high (higher than 60% of total fatty acids). As Table 1 shows, saturated fatty acids contained in the oils are only small amount of palmitic acid (6.3%) and barely traceable amount of stearic acid. *Perilla* is grown to produce seed oils as well as leaves which is one of most popular green vegetables consumed in Korea. Such high nutritional value of the vegetable oils makes *Perilla* as an attractive target to genetically modify lipid production qualitatively as well as quantitatively.

Lipids are loosely defined as compounds soluble in nonpolar organic solvent such as chloroform. Under this definition, substances in this catagory are very diverse in structure. However, lipids in the form of fatty acyl glycerol including triacylglycerol is most abundant. Triacylglycerols are the major forms of photosynthetates stored in seeds of many different plant species. In some plant species, lipids constitute more than 60% of dry weight of seeds.

Modification of lipid production in plants using biotechnology has been persued since early '90s. Most of

these research endeavors aim at the change of fatty acid composition such as the production of shorter or longer chain fatty acids and the production of more unsaturated or more saturated fatty acids. Fatty acids have been exploited extensively not only for human consumption but also for industrial uses. As Ohlrogge summerized examples of industrial use of fatty acids and their derivatives, approximately one-third of vegetable oils in the world are used in non-food industry [12]. Short to medium chain fatty acids such as lauric acid (12:0) are utilized in producing soups, detergents and surfactants, while erucamide, which is extensively used in plastic film manufacture, is converted from erucic acid (22:1), a major constituent of *Brassica napus* seeds. Other examples of industrial use of fatty acids and their derivatives includes vernolic acid (epoxy), ricinolic acid (hydroxy) and linolenic acid (trienoic). Using such fatty acids, plasticizers, lubricants, paints and vanishes are produced.

In order to manipulate lipid production in plants by genetic engineering, it is prerequisite to fully understand biosynthetic pathway of fatty acid and lipids [2, 12, 13]. Biosynthesis of lipids occurs in the plastids and in the endoplasmic reticulums. In the first step of fatty acid synthesis which takes place in the plastid, malonyl-CoA is produced from acetyl-CoA and CO2 by the action of acetyl-CoA carboxylase. Then malonyl-CoA:ACP transacylase transfer malonyl moiety from malonyl-CoA to an acyl carrier protein (ACP). In the first round of condensation reactions leading to the synthesis of fatty acids, acetyl-CoA is condensed with malonyl-ACP to produce 3-ketobutyryl-ACP by 3-ketoacyl-ACP synthase Ⅲ(KAS Ⅲ). Through a consecutive action of 3-ketoacyl-ACP reductase, 3-hydroxylacyl-ACP dehydrase and enoyl-ACP reductase, the first round of the condensation reaction is completed in that butyryl-ACP is produced from 3-ketobutyryl-ACP. In the following rounds of fatty acid chain elongation reaction, malonyl-ACP donates 2 carbon unit to the growing fatty acid chain esterified to ACP. The condensation reaction of malonyl-ACP to fatty acyl-ACP is catalyzed by two different 3-ketoacyl-ACP synthases other than KAS Ⅲ. During the chain elongation of short to medium chain fatty acyl-ACP, KAS Ⅰ is known to be involved while KAS Ⅱ catalyzes the condensation reaction of malonyl-ACP to longer chain fatty acyl-ACP (longer than palmitoyl (16:0)-ACP). During and/or after the fatty acyl chain elongation process, acyl-ACP thioesterase dissociates fatty acids from ACP and produce fatty acyl-CoA. Fatty acyl-CoA is then translocated to the endoplasmic reticulums (ER) where membrane lipids and other lipids such as triacylglycerol are assembled. Assembly of fatty acyl chain and glycerol in plants is achieved by two different types of cellular pathways, namely prokaryotic and eukaryotic pathway. In the prokaryotic pathway, acyl-ACP is a substrate for the assembly, and palimtate is predominantly esterified to sn2 position of glycerol backbone. On the other hand, the eukaryotic pathway, which occurs in the ER, uses acyl-CoA as a substrate and 18 carbon fatty acid is esterified to the sn2 position. In the modification reaction of fatty acid such as desaturation, diacyl glycerols are used as substrates except in the reaction to produce oleic acid from stearic acid which use stearoyl-ACP as a substrate.

Even though lipids (triacylglycerols) are the major constituent of some plant seeds, such seeds also accumulate considerable amount of starch as storage substances. Starch biosynthetic pathway is considered directly or indirectly interconnected with lipid biosynthetic pathway. Therefore, it would be necessary to understand overall carbon metabolism for the genetic manipulation of lipid production of plants. Starch synthesis in plants generally occurs in two different types of plant tissue which are source and sink tissue. In source tissue, starch is a transient storage of photosynthetates prior to translocation of them to sink tissue, while sink tissue accumulate starch for longer storage. The photosynthetic carbon reduction cycle produces three carbon compounds such as phosphoglyceric acid which are in turn converted into glucose by conjugation. On the other hand sucrose is generally the major form of photosynthetates translocated which is digested into UDP-glucose and fructose by the action of sucrose synthase. Glucose phosphate is the primary substrate in the reaction leading to the production of starch in that ADP-glucose pyrophosphorylase (AGP) produces ADP-glucose from glucose-1-phosphate and ATP. ADP-glucose is the substrate of starch synthase which catalyze the reaction to form $\alpha 1 \rightarrow 4$ glycosidic linkage between glucosyl moieties. After a series action of branching enzymes and debra- nching enzymes, starch is produced.

We have cloned and characterized several genes involved in fatty acid synthesis from *Perilla* as well as from other plant species. We also have cloned three AGP cDNAs to increase lipid production of *Perilla* by perturbing carbon flow in developing Perilla seeds. We transformed and are transforming *Perilla* with some of

these genes in order to genetically manipulate lipid production of the plant seed. Particulary, we found that transgenic *Perilla* leaves expressing △15-desaturase under control of 35S promoter contain increased amount of α-linolenic acid.

Isolation of genes involved in fatty acid synthesis

Genes, whose products catalyze steps in the biosynthesis of plant compounds, have been extensively isolated recently. By having these genes in hand, it is now possible to study the biochemical properties of a biosynthetic metabolism at the level of molecular biology. Moreover, it also provides us an opportunity to genetically modify biosynthetic pathways in transgenic plants.

As mentioned above, considerable proportion of vegetable oils are used in nonfood industry. Of these, short to medium chain fatty acids such as lauric acid (12:0) are utilized in producing soups, detergents and sufactants. Several plant species, including *Iris* sp., California Bay and *Cuphea* sp are known to produce short to medium chain fatty acids (Table 1) [3, 14]. Therefore, genes responsible for the production of short to medium chain fatty acid in these plants drew much attention from biochemists and molecular biologists and were isolated [8, 18].

Fatty acid synthesis in plants occurs in the plastids. It is a complex metablic pathway in which many different enzymes are involved [13]. What makes the pathway more complicated is the existence of several different isozymes participating in similar biochemical reactions. For example, at least three different types of 3-ketoacyl-ACP synthase (KAS) are considered to be involed in the biosynthesis of stearic acid(18:0). KASⅢ catalyzes the first condensation reaction in the fatty acid biosynthesis [6]. In early cycles of fatty acid chain elongation, KAS Ⅰ is responsible for the condensation reaction betweeen malonyl-ACP and shorter chain fatty acyl-ACPs while KAS Ⅱ is invloved in the chain elongation of longer fatty acids. It was recently reported that transgenic *Arabidopsis* coexpressing KASA1 gene isolated from *Cuphea wrightii* and *Cuphea* FatB gene produce shorter chain fatty acids [10].

The chain length of a fatty acid is also determined by substrate specificities of acyl-ACP thioesterases. Seeds of California Bay contain laurate (12:0) as a major fatty acid components and possess high levels of 12:0 acyl-ACP thioesterase [14]. Critical role of the thioesterase in determining the chain length of fatty acid was also evidenced by an experiment using transgenic plants in that seeds of transgenic *Arabidopsis* expressing 12:0 acyl-ACP thioesterase gene from California Bay produced up to 25% lauric acid in their seed oils [18]. Another example of plant species accumulating shorter chain fatty acid in seeds is the Mexican shrub *Cuphea*. Cuphea seeds are abundant in 8:0 and 10:0 fatty acid [3]. From the *Cuphea* cDNA library, Jones et al. isolated several thioesterase genes of which one encodes a 16:0 specific enzyme [7]. From parsimony analysis of a number of plant acyl-ACP thioesterases, they catagorized thioesterases into two classes, namely *FatA* and *FatB*. *FatA* and *FatB* are different each other not only in sequence but also in function. *FatA* genes are known to encode thioesterases specific for 18:1 substrate. On the other hand, thioesterases encoded by *FatB* genes tend to be specific for shorter chain fatty acyl-ACP (C14 to C18 or less). Therefore, KAS and thioesterase genes would be two main targets for the

Table 1. Composition of fatty acids in total lipids extracted from several plant species.

Plant (Source)	TFA[1]	Fatty acid composition (%)								
		8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2	18:3
Perilla frutescence (Seeds)	400.1					6.3	t[2]	20.1	13.4	60.2
Iris germanica (Roots)	31.19	0.7	3.2	15.5	57.0	7.9	0.6	2.8	11.0	1.3
Iris tectorum (Roots)	47.17	0.8	1.3	0.8	75.9	8.7	0.5	0.5	9.3	2.2

[1]TFA: total fatty acid (mg/g dry weight tissue)
[2]t : trace amount

Table 2. List of cDNA clones involved in fatty acid biosynthesis

Gene	Plant	Length (# of a.a)	Function	Genbank Acc. No.
Pffad7	*Perilla frutescens*	1797 (438)	△15 desaturase, Plastidial	U59477
Pffad3	〃	1620 (413)	△15 desaturase, ER specific	
			Acyl-ACP thioesterase	
PfFatA1	*P. frutescens*	1466 (368)	($C_{18:1}$-ACP → $C_{18:1}$)	L78468
ItFatA1	*Iris tectorum*	1416 (371)	($C_{18:1}$-ACP → $C_{18:1}$)	L78467
ItFatB1	〃	1639 (427)	($C_{16:0}$-ACP → $C_{16:0}$)	
ItFatB2	〃	1832 (425)	〃	
IgFatA1	*Iris germanica*	1460 (364)	($C_{18:1}$-ACP → $C_{18:1}$)	
IgFatB1	〃	1708 (427)	($C_{16:0}$-ACP → $C_{16:0}$)	
IgFatB2	〃	1859 (425)	($C_{16:0}$-ACP → $C_{16:0}$)	
			3-Ketoacyl-ACP Synthase	
KAS Ⅲ*A*	*P. frutescens*	1906 (401)	(C_2 → C_4)	AF026150
KAS Ⅲ*B*	〃	1904 (400)	〃	AF026151
KAS Ⅰ	〃	1841 (474)	(C_4 → C_{16})	AF026148
KAS Ⅱ	〃	2072 (530)	(C_{16} → C_{18})	AF026149

genetic engineering toward the production of short chain fatty acids.

We have cloned four different KAS genes from *Perilla frutescence* (Table 2). For this, degenerated primers were synthesized, and several fragments were amplified by PCR. PCR fragments were used as probes to clone cDNAs encoding 3-ketoacyl-ACP synthase. Sequence analysis revealed that two of four cDNA clones encode KASⅢ (KASⅢA and KASⅢB) while the other two encode KASⅠ and KASⅡ, respectively. KASⅠ cDNA clone is 1841bp long of which ORF encodes a polypeptide conssisting of 474 amino acids. KASⅢA and Ⅲ B genes are 1906 and 1904 bp long and encode peptides of 401 and 400 amino acids, respectively. Nucleotide length of KASⅡ gene is 2072bp and a peptide of 530 amino acid is encoded.

We also have cloned six genes encoding acyl-ACP thioesterases from *Perilla frutescence, Iris tectorum and Iris germanica.* These seven *Fat* genes fell into two classes as revealed by sequence analysis, in that three *Fat* genes are catagorized in *FatA* class while the other four are the members of *FatB* class. Nucleotide sequences of two *FatB* genes from *Iris germanica* (*IgFatB1* and *IgFatB2*) were also analyzed. *IgFatB1* and *IgFatB2* consist of 1708 and 1859bp and encode peptides of 427 and 425 amino acids, respectively. In order to test substrate specificity, *IgFatB1* and *IgFatB2* genes were expressed in *E. coli* [19]. For this, *E. coli* expression vectors containing the thioesterase genes were constracted so that the thioesteases were fused to hitidine tag. Fig. 1 shows SDS-PAGE profiles of total protein extracts from *E. coli* and the thioesterase partially purified by Nickel column chro-

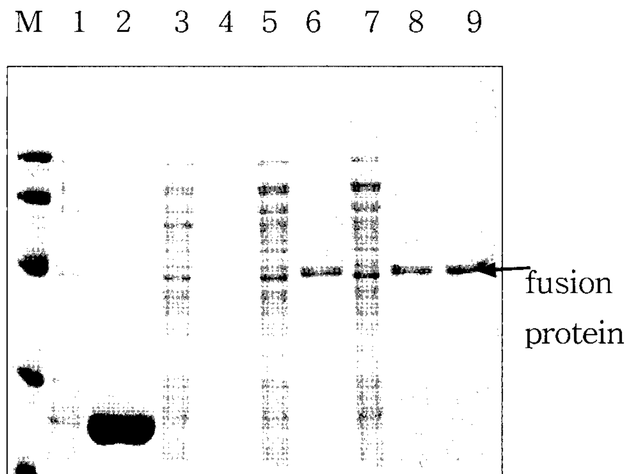
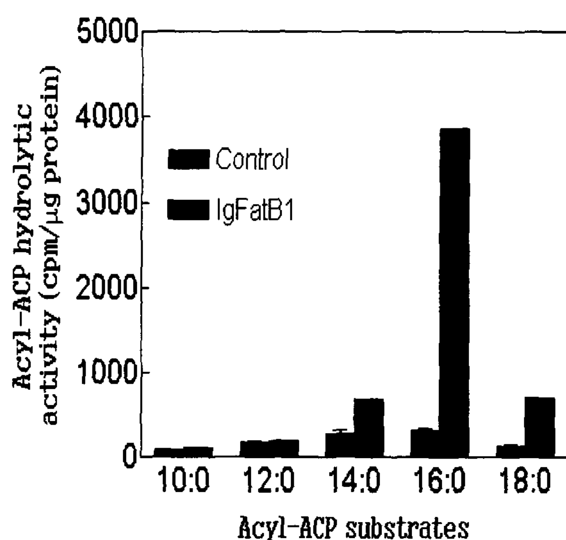


Figure 1. SDS-PAGE profiles of crude protein extract and acyl-ACP thioesterase purified from *E. coli* expressing *IgFatB1*. M: marker; 1, 2: positive control (pQE16); 3, 4: without IPTG induction; 5 to 9: with IPTG induction; 1, 3, 5, 7: Crude extract; 2, 4, 6, 8, 9: acyl-ACP thioesterase purified by nickel column chromatography.

matography. As shown in Fig. 2, thioesterases encoded by *IgFatB1* and *IgFatB2* are stearoyl-ACP specific. As reported in recently published paper, it could be possible that short chain fatty acids are produced by the coexpression of *FatB* and *KAS I* genes in transgenic plants [10]. we have plan to construct a transformation vector harboring a *Perilla KAS I* gene and a *Iris FatB* gene and to transform *Perilla* with these genes for the production of short to medium chain fatty acids.

A



B

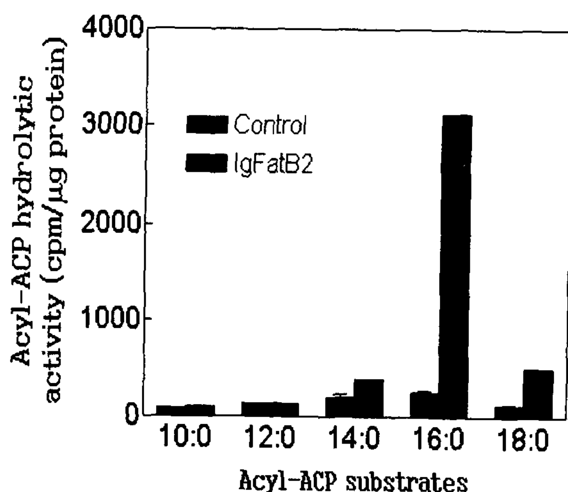


Figure 2. Thioesterase activities and substrate specificities of *IgFatB1* (A) and *IgFatB2* (B) gene products produced in *E. coli.*

Isolation and Characterization of genes encoding $\Delta 15$ acyl-ACP desaturase

Vegetable oils have gradually replaced animal fats as the major source of lipids in human diets. Because content of saturated fatty acids in vegetable oil is far less than in animal fat, this replacement is considered to be beneficial in reducing cholestrol level. Especially, α-linolenic acid (ALA, 18:3), which is the major fatty acid constituent of *Perilla* seed oils, was reported to have several positive effects on human health. Such effects include the reduction of cholesterol level in blood and of arthritis, the decrease of blood pressure, and the enhancement of immune systems and brain activity [9, 16]. However, the stability of vegetable oils during storage is negatively affected by the presence of highly unsaturated fatty acids in vegetable oils. Lipoxygenation of unsturated fatty acid during storage renders foul flavor to vegetable oils. Therefore, biotechnological research to modify fatty acid composition of vegetable oils is directed either to increase or to decrease highly unsaturated fatty acid content.

During and after the synthesis of fatty acid, modification reactions to produce unsaturated fatty acids take place in the plastids (prokaryotic pathway) as well as at the endoplasmic reticulums (eukaryotic pathway) [5]. From biochemical and genetic characterization of fatty acid desaturation reaction in plants, it was revealed that at least eight desaturase genes (*Fab2, Fad2 to Fad8*) were identified in *Arabidopsis* to be involved in these processes [2, 13]. After seven condensation reactions between malonyl-ACP and fatty acyl-ACP, stearoyl-ACP is produced. At this point, the first double bond is introduced to fatty acid by stearoyl-ACP desaturase (*Fab2*) to produce oleoyl-ACP (18:1). Further desaturation reactions of fatty acids occur after oleic acid is esterified to phosphatidyl glycerol. In prokaryotic pathway of lipid synthesis, phosphatidic acids are assembled in the way that either oleic acid or palmitic acid is esterified to sn1 position of glycerol-3-phosphate and palmitic acid is esterified to sn2 position. Then head group activation take place to produce membrane lipids such as phosphatidyl cholin and mono(di-)-galactosyl diacylglycerols. In prokaryotic lipid desaturation, five *fad* genes were identified. Of these, products of *fad4* and *fad5* genes were reported to catalyze desaturation of palmitic acid at sn2 position of the lipids. Oleic acid, which is esterified to sn1 position of glycerol, desaturated by *fad6* gene products yielding linoeic acid (18:2). α-Linolenic acid is produced

from linoleic acid by the action of plastidial △15-desaturases (FAD7 and FAD8).

From *Perilla* cDNA library, we have cloned two different △15-desaturase genes (*Pffad7* and *Pffad3*)(Table 2). To obtain △15-desaturase cDNA clones, degenerated primers were designed based on information from nucleotide sequence analysis of known △15-desaturase genes, and a 269bp fragment was amplified by PCR. This fragment was then used as a probe to clone the genes. Nucleotide sequences of cloned *Pffad7* and *Pffad3* cDNA clones were then aanlyzed. By comparing nucleotide sequence homology, we concluded that the one is an ER specific desaturase (Pffad3) and the other is a plastid specific desaturase (Pffad7). Pffad3 and Pffad7 consist of 1620 and 1797 nucleotide base pairs and encode peptides of 413 and 438 amino acids, respectively.

Using *fad3* and *fad7* cDNA clones, tissue specific expression of △15- desaturase genes was analyzed by Northern blot hybridization (Fig. 3) *Fad7*, encoding the plastid specific △15-desaturase, was extensively expressed in leaves, while mRNA of *fad7* was barely detected in roots and seeds. On the other hand, the ER specific △15-desaturase gene, *fad3*, expressed considerably in leaves as well as in seeds. Expression of *fad3* during the seed development was then monitored as shown in (Fig. 4). As the figure shows, *fad3* was expressed throughout the seed development except in the seeds at very early developmental stage (week 1) and at mature dry stage. This observation is well correlated with changes in fatty acid content of Perilla seeds during seed development (Fig 5). For the analysis of fatty acid contents of Perilla seeds, seeds at four different developmental stage were harvested (from day7 to day29 after flowering). As the figure shows, ALA content of Perilla seeds increased steadily with the progress of seed developement indicating that the enzyme (△15-desaturase) responsible for the production of ALA is engaged to work throughout seed development tested.

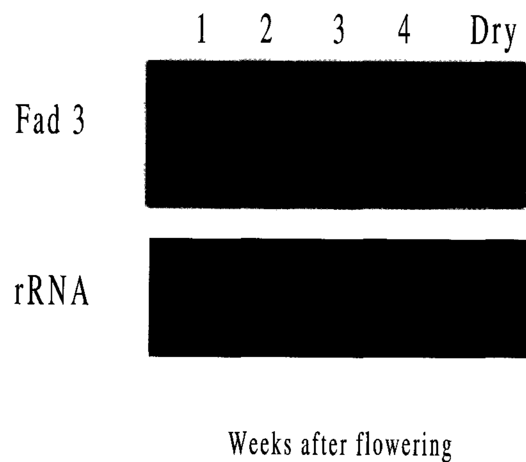


Figure 4. Expression of the ER specific △15-desaturase gene (*fad3*) during seed development as monitored by Northern blot analysis

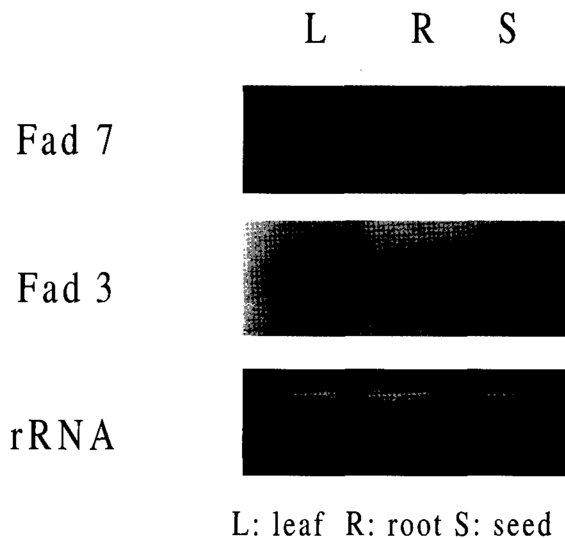


Figure 3. Tissue specific expression of two Perilla △15-desaturase genes (*Pffad7* and *Pffad3*) as monitored by Northern blot analysis. Fad7: *Pffad7*, Fad3: *Pffad3*

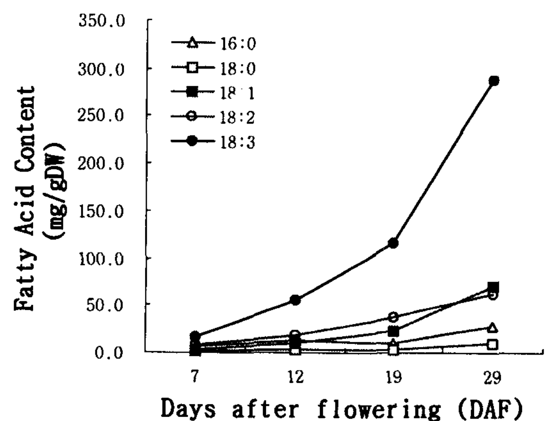


Figure 5. Changes in fatty acid contents of *Perilla* seeds during seed development

Table 3. Summery of optimal protocol for the transformation of *Perilla frutescence*

	Media	Hormone	Antibiotics	Treatment Period
Explant	Young cotyledons (2 3 days after germination)			
Pretreatment	MS	-	-	3 hr
Coculture	MS	3mg/L BAP	-	2 days
Shoot Induction	MS	0.5mg/L BAP	125 mg/L kanamycin 500 mg/L carbenicillin	3 weeks
Root Induction	1/2 MS	-	500 mg/L carbenicillin	10 days

Perilla transformation with Δ15 acyl-ACP desaturase gene

Perilla frutescence is an oil seed crop widely grown in Korea. As described above, vegetable oils from *Perilla* seeds have high α-linolenic acid which represent the high nutritional value [9, 16]. Since *Perilla* is such an exellent oil seed crop, it is a good target plant for the genetic manipulation of its lipid production. To achieve this, it is prerequisite to optimize transformation protocols. Related to this, only a couple of research papers on the tissue culture of *Perilla* was published [15]. In order to develop a transformation protocol using *Agrobacterium*, a series of experiments was performed to choose suitable explants for shoot regeneration, optimal concentrations of growth hormone and optimal strength of selection pressure to select transformants. As described in (Table 3), very young cotyledons (2 days after germination) turned out to be the best explants, and 0.5mg · L^{-1} BAP was the optimal concentration for direct shoot regeneration. We observed higher rate of shoot regeneration from young cotyledon explants in the presence of 3mg · L^{-1}BAP. However, shoots developed under this condition were very poor in root developement when those shoots were transferred to root developing media. In our transformation experiments, *NPTII* was used as a selectable marker gene. For the selection of transformants, 125mg · L^{-1} kanamycin was added to shoot regeneration media.

In order to genetically modify α-linolenic acid content in *Perilla* lipids, we constructed transformation vectors as shown in Fig. 6. *Pffad7* cDNA was under control of 35S promoter and *NPTII* gene was used as a selectable marker. After cotyledons were infected by *Agrobacterium*, transgenic shoots and roots were successibly induced to form as shown in Fig. 7. Putative transgenic plants were then grown in a green house and transgenic *Perilla* was identified by PCR. α-Linolenic acid content in *Perilla* lipids were then analyzed for leaves and seeds. As shown in Fig. 8, α-linolenic acid of lipids extracted from leaves of transgenic *Perilla* increased by 10%. However, α-linolenic acid of the transgenic *Perilla* seed oils considerably decreased. Pffad7 encodes the plastid specific $\triangle$15-desaturase which was barely expressed in seeds of non transgenic Perilla (Fig. 3). Therefore, it may be possible that expression of plastid specific $\triangle$15-desaturase perturbs lipid synthesis pathway in transgenic *Perilla* seed resulting in the decrease of α-linolenic acid in the seed oils. To test this possibility, we constructed another Perilla transformation vector containing *Pffad3* gene under vicillin promoter (Fig. 6). Currently, we are transforming *Perilla* with this gene. After the transgenic plants are obtained, we will analyze fatty acid composition of the *Perilla* seed oils.

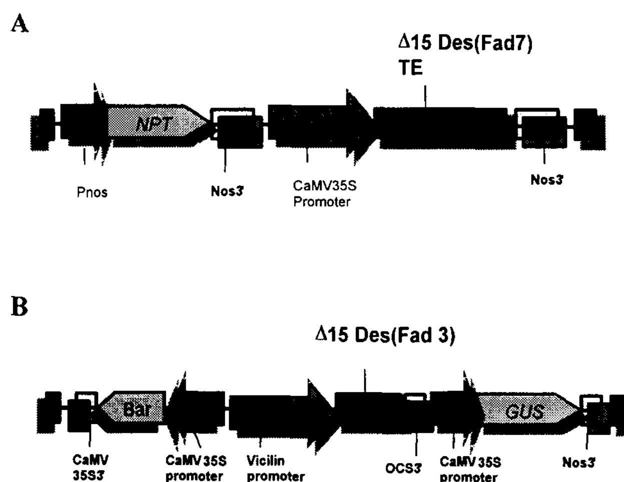


Figure 6. Construction of T-DNAs containing $\triangle$15-desaturase genes for the transformation of *Perilla frutescence*. A. *Fffad7* gene under 35S promoter control; B. *Pffad3* gene under seed specific vicillin promoter control.

Cloning of Perilla ADP-glucose pyrophosphorylase and their expression in *E. coli*

Carbon metabolism in storage tissue is not straight forward. The fate of photosynthetates translocated to storage tissue, such as seeds of monocots and docots, potatotubers and fruits, would be very diverse in develpoment as well as tissue type specific manners. For example, the main storage form of potato tuber and seed endosperm is starch, while cotyledons of Perilla accumulate triacylglycerols as a main storage compound.

Sucrose, a major form of photosynthate translocated to sink tissue, enters storage cells and digested to UDP-glucose and fructose by sucrose synthase. UDP-glucose pyrophosphorylase then convert UDP-glucose to glucose-1-phosphate which in turn is converted to glucose-6-phosphate. Glucose phosphate and fructose enter various metabolic pathway occurring in cytoplasm and the plastids. Pathways of carbon metabolism in sink tissue interconnected each other directly or indirectly. Therefore, it is quite possible that genetic manipulation of a carbon metabolism affect carbon flow to other metabolic pathways.

It was attemped to increase starch production in sink tissue by genetic engineering of ADP-glucose pyrophosphorylase (AGP) gene [17]. Since AGP catalyzes the rate limiting step in starch synthesis and functions to control carbon metabolism, AGP activity is tightly controlled by cellular energy state rather than the level of AGP gene epression. Plant AGP is activated by the presence of 3-PGA and inhibited by inorganic phosphate. For the manipulation of AGP activity in transgenic plants, it

Figure 7. Photographic demonstration of the transformation of *Perilla frutescence*. A. Shoot regeneration of kanamycin resistant shoots from cotyledonary explants. B. Induced root formation from regenerated shoots. C. Transgenic *Perilla* grown in a green house.

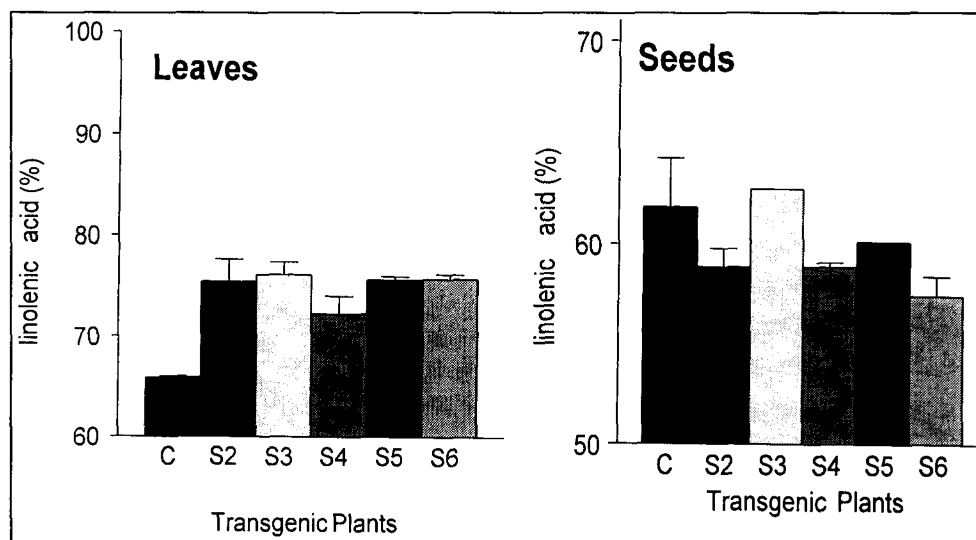


Figure 8. Content of α-linolenic acid in lipids extracted from transgenic and nontransgenic *Perilla frutescence* leaves (left) and seeds (right). C. nontransgenic control. S2 to S6: transgenic plants (To)

is necessary to obtain mutated AGP genes of which product show different sensitivity to activators and inhibitors. Currently several mutant genes from bacteria as well as from plants are available [1, 4]. AGPs encoded by those genes are more sensitive to activators and less. sensitive to inhibitors.

Sunflower (*Helianthus annuus* L.) accumulates starch transiently at early stage of seed development and the lipid synthesis takes place at later stage of seed development [11]. Even though it is not certain whether carbon flow leading to lipid synthesis in those seeds passes through starch synthetic pathway, at least considerable proportion of starch accumulated in those seeds transforms into lipids. This previous observation may indicate that lipid productivity of seeds could be enhanced by increasing the transient accumulation of starch. In order to test this hypothesis, we cloned three Perilla AGP genes from immature seed specific cDNAs library. As nuclotide sequence analysis revealed, two genes(*Psagpl* and *Psagp2*) encode AGP small subunits while the other (*Plagp*) encodes AGP large subunit. All of them appear to contain regions encoding transit peptide for the plastid targetting. In order to express these genes in *E. coli*, we amplified truncated AGP genes encoding putatively mature peptides and coexpress the truncated AGP genes with potato AGP gene. The expression of *Perilla* AGP genes in E. coli was then monitored by the Western blot analysis and AGP activity assay (Fig. 9, Table 4). As Fig. 9 shows, truncated PSAGP1 and PSAGP2 presented in soluble protein extracts from *E. coli* coexpressing *Perilla* AGP small subunit gene and potato AGP large subunit gene. This result indicates that *Perilla* AGP small subunits form active heterotetramers with potato AGP large subunit. These are also evidenced by the analysis of AGP activity. As Table 4 shows, crude extracts from *E coli* coexpressing *Perilla* AGP small subunit/potato AGP large subunit genes show considerable level of AGP acitivity. On the other hand, crude extracts from *E coli* coexpressing *Perilla* AGP large subunit/potato AGP small subunit genes show no AGP acitivity.

In order to test whether upreg mutated AGP (*upregl* and *upreg2*) large subunit gene from potato could be utilized in genetic manipulation of *Perilla* AGP activity in seeds, we coexpressed *upregl* and *upreg2* genes with *Perilla* AGP small subunit genes. As Fig. 10 shows, *E coli* coexpressing *Perilla* AGP small subunit and potato *upreg* mutant genes accumulate more starch than coexpressing *Perilla* AGP small subunit/wild-type AGP large subunit genes. This result indicate that the mutant phenotype of upreg mutant gene is maintained when coexpressed with *Perilla* AGP small subunit gene. We are currently constructing *Perilla* transformation vectors containing the AGP *upreg* mutant under control of vicillin promoter. After we obtain transgenic *Perilla,* lipid production of seeds will be examined.

Table 4. Specific ADP-glucose pyrophosphorylase (AGP) activity in crude extracts from *E. coli* coexpressing plant AGP genes

Plasmids coexpressed[2]	Specific Activity[1]
pDKS1/pDKL	n.d.
pDKS1/pML7	0.02
pDKS2/pML7	0.01
pML10/pML7	0.4

[1]Specific Activity: *mumolPi/min/mgProtein*
[2]pDKS1, pDKS2, pDKL: E. coli expression vectors containing Psagp1, Psagp2 and Plagp, respectively.
pML10, pML7: E. coli expression vectors containing potato AGP small and large subunit genes, respectively

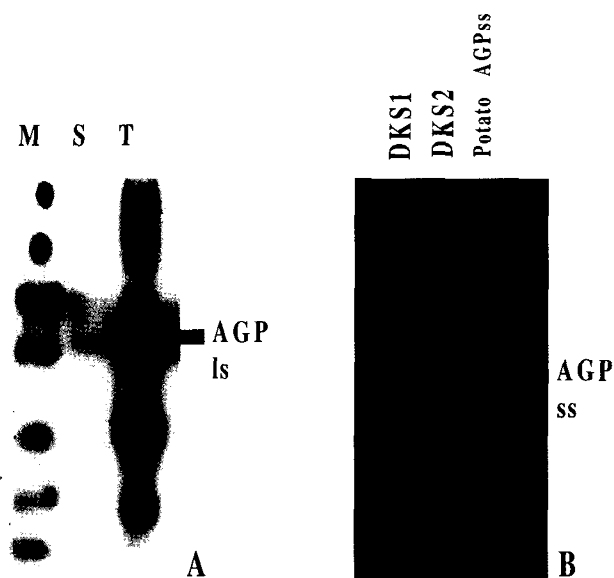


Figure 9. Western blot analysis of Perilla ADP-glucose pyrophosphorylase (AGP) expression in *E. coli*. A. Western blot analysis of AGP large subunit. B. Western blot analysis of AGP small subunit. M. molecular marker, S. soluble proteins, T. total proteins.

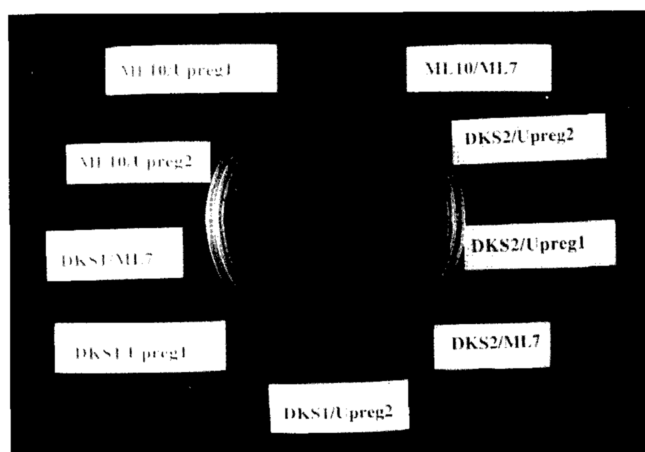


Figure 10. Comparison of starch accumulation in *E. coli* coexpressing different combination of ADP-glucose pyrophosphorylase small and large subunit genes.

Concluding Remarks

Examples of successful genetic manipulation of plant oil production has been accumulated continuously. Efforts, which have been made to manipulate the oil production, aim at the enhancement of either industrial value or nutritional value of vegetable oils. In this sense, researches have been concentrated on modifying fatty acid chain length and fatty acid unsaturation. We have been working on the lipid production of *Perilla frutescence*. We isolated genes involved in synthsizing and desaturating fatty acids. Currently, we are transforming *Perilla* and sesame with those genes. By doing this, we hope to develop a new cultivar producing vegetable oils with enhanced industrial and/or nutritional quality. We also isolated AGP genes from *Perilla* and observed that AGP *upreg* mutant genes from potato could form active AGP enzyme with mutant phenotype. We are currently performing experiments to transform *Perilla* with *upreg* mutant genes. It may be possible to encrease oil productivity of *Perilla* seeds by expressing the *upreg* mutant gene in *Perilla* seeds.

Production of storage oil in plant seeds is not straight forward. Many different metabolic pathways are directly or indirectly interconnected with lipid biosynthetic pathway. Moreover, the accumulation of oil droplets in oil-body implicates complex cellular process leading to the biogenesis of cellular organelles. Therefore, we need to accumulate more detailed information on biochemical, moelcular and cellular biological properties of lipid production in plants. To achieve this, genes involved directly or indirectly in the process should be isolated and the cellular function of those genes should be scrutinized. One strategy to access would be to analyze phenotypic properties of each transgenic plant. On the basis of these accumulated information, we may be possible to genetically manipulate lipid production of plants qualitatively as well as quantitatively.

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