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*FAD*2-silencing has pleiotropic effect on polar lipids of leaves and varied effect in different organs of transgenic tobacco

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Abstract

A microsomal ω-6 fatty acid desaturase gene (*FAD*2) of tobacco (*Nicotiana tabaccum*) was cloned by PCR-based method and a partial coding sequence of the putative *FAD*2 gene was used to create intron-containing construct expressing hairpin RNA for silencing endogenous *FAD*2 gene. In addition to a marked increase of oleic acid in phosphatidylcholine and phosphatidylethanolamine, the main lipid components of the extrachloroplastic membranes of plant cells, the silencing of *FAD*2 resulted in pleiotropic effect on polar lipids of leaves, i.e., a significant increase of oleic acid levels in sulfoquinovosyldiacylglycerol, phosphatidylglycerol, digalactosyldiacylglycerol and monogalactosyldiacylglycerol, located predominantly in the chloroplast, and a significant reduction of palmitic acid levels in some individual polar lipids. The significant increase of oleic acid only found in lipids of leaves and seeds of transgenic lines proved the diversity of the silencing effect in different organs. The possible mechanisms involved in the control of lipid unsaturation level in different organs of transgenic tobacco were discussed.

Keywords: Gene silencing; *FAD*2; Lipid; Tobacco; Organ

1. Introduction

Plant oils represent a vast renewable resource of highly reduced carbon and those with a high content of oleic acid are of interest for nutritional and industrial purposes [1]. In fact, oils rich in monounsaturated fatty acids and poor in palmitic acids are more suitable and beneficial for improved oil stability, flavor, and nutrition [1]. A thorough understanding of desaturases involved in lipid synthesis is necessary to cultivate crops with oils containing suitable fatty acids. However, isolation and biochemical characterization of most fatty acid desaturases has proven difficult due to their membrane-bound nature that a considerable amount of knowledge about plant desaturases comes from the characterization of a series of *Arabidopsis* mutants with defects in fatty acid desaturation [2].

There are two distinct pathways (''prokaryotic'' pathway, ''eukaryotic'' pathway) in plant cells for the biosynthesis of glycerolipids and the associated production of polyunsaturated fatty acids [2,3]. Two pathways coordinate in glycerolipid synthesis in plants and the balance of fluxes through these pathways may be altered to compensate for the effects of mutations that block steps in one of the pathways [2,4]. At least for the endoplasmic reticulum (ER) and the plastid, lipid traffic between the membranes is bi-directional and most of the mutations affect the composition of both chloroplast and extrachloroplast membranes even though the enzymes are located in one compartment or the other [4]. Genes of lipid synthesis have been cloned in many plant species among which the ω-6 desaturase gene is of particular interest for it is the enzyme that places the second double bond in the fatty acid and catalyzes the first step in polyunsaturated fatty acid biosynthesis. All higher plants contain one or more microsomal ω-6 desaturase(s) (also named microsomal Δ12 desaturase, oleoyl-PC desaturase or *FAD*2) that insert a double bond between carbon 12 and 13 of monounsaturated oleic acid to generate polyunsaturated linoleic acid and control the most of polyunsaturated lipid synthesis in nonphotosynthetic plant tissues [5,6]. The *FAD*2 gene appears to be also important in the chilling sensitivity of plants, as polyunsaturated membrane phospholipids are important in maintaining cellular function

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and plant viability at low temperatures [6–8]. Many details about *FAD*2 remain elusive, since *FAD*2 is an ER membrane-bound desaturase [9,10], such as the relations between prokaryotic and eukaryotic pathways, the regulation of *FAD*2 activity in a wide range of environmental conditions, the development- and tissue-specific *FAD*2 modulation etc. It has been demonstrated that hpRNA-mediated gene silencing, a kind of post-transcriptional gene silencing (PTGS), results in high efficiency and efficacy of gene silencing in plants [11–13]. A clearer understanding of the expression patterns of the *FAD*2 gene and associated fatty acid composition in *FAD*2-silenced plant cells is crucial in understanding the function of *FAD*2 gene, and in applying such a PTGS technology to manipulate the polyunsaturated fatty acid composition of plant membranes, predictably to improve the seed oil value, vigor and viability of crop plants.

In this report, we used a partial coding sequence of tobacco (*Nicotiana tabaccum*) *FAD*2 gene to make an hpRNA-producing construct to specifically silence endogenous *FAD*2 gene. We demonstrated that the silencing of *FAD*2 gene had pleiotropic effect on fatty acid composition of lipids in transgenic plants. To our knowledge, this is the first report on the tissue-specific effect of *FAD*2 gene silencing in transgenic plant.

2. Materials and methods

2.1. FAD2 cloning

A list of *FAD*2 sequences identified from different plant species was aligned to design a pair of degenerated oligonucleotide primers. The sense primer was 5′-CGTCGCCA (CT) CA (CT) TC (CT) AACAC-3′, and the antisense primer was 5′-CCCCTAA (AG) CCA (AG) TCCCA (CT) TC-3′. A 452 bp sequence was amplified by PCR from tobacco (Wisconsin 38) cDNA prepared from leaves. The procedure of PCR is 5 min at 94 °C, 35 cycles for 30 s at 94 °C, 45 s at 54 °C, 1 min at 72 °C, and extension 10 min at 72 °C. Both 3′-RACE and 5′-RACE were performed using RACE kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

2.2. Gene-silencing constructs

The 452 bp *FAD*2 fragment was inserted into pKANNIBAL vector in inverted repeat configuration essentially as described [11]. The correct orientation of silencing construct was confirmed by sequencing. A fragment containing the silencing construct was subcloned as *Not*I fragments into binary vector pART27 [14] as shown in Fig. 2, and then introduced into *Agrobacterium tumefaciens* strain LBA4404 by tri-parental mating for transformation of tobacco by the leaf-disc method [15]. Seedlings that survived in MS medium containing kanamycin (150 mg/L) were grown in growth chamber under 16 h 80 μmol m^{-2} s^{-1} light/8 h dark conditions at 24 °C.

2.3. Southern blot analysis

Tobacco genomic DNA was isolated from T0 generation and wild-type seedlings according to Porebski et al. [16]. Approximately 20 μg of DNA was digested with *Eco*RI and separated on a 0.8% (w/v) agarose gel by electrophoresis and transferred to a Hybond N^+ nylon membrane according to Sambrook et al. [17]. Southern blot analyses were carried out by hybridizing with a [^{32}P]dCTP-labeled *FAD*2 452 bp fragment got by PCR using the purified *FAD*2 cDNA 452 bp fragment as template. Prehybridization and hybridization was carried out using hybridization cocktails I and III (Sangon, Shanghai, CN) following the manufacturer's instructions.

2.4. RT-PCR

RNA was prepared from tissues of tobacco using the Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. After digestion with RNase-free DNase I (Promega, Madison, WI, USA), the reverse transcription was carried out using reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The PCR procedures were same to that used in *FAD*2 cDNA 452 bp fragment cloning, except that the repeat cycles were 25 instead of 35. Tobacco actin Tac9 was used as a parallel control.

2.5. Northern blot analysis

Fifteen micrograms of total RNA was separated on a denaturing formaldehyde gel and transferred to Hybond N^+ nylon membrane (Pharmacia) according to Sambrook et al. [17]. The probes used for *FAD*2 mRNA were the same to that used in Southern blot analysis. To confirm that lanes were equally loaded, nylon membrane was stripped by washing in 10 mM Tris–Cl (pH 7.4), 0.2% (w/v) SDS at 75 °C for 1 h, then re-hybridized with an α-^{32}P dCTP-labeled Tac 9 probe. The hybridization was essentially the same as described in Southern blot analysis section.

2.6. Fatty acid analysis

Lipids were extracted from different tissues of tobacco according to the method of Bligh and Dyer [18]. The individual lipids separation and the fatty acid analyses were carried out according to the method of Xu et al. [19]. Relative fatty acid compositions were calculated as the percentage that each fatty acid represented of the total measured fatty acids. An additional indirect method of assessing the cumulative effects of *FAD*2 activity during leaf fatty acid synthesis is through an oleic desaturation proportion (ODP) parameter as described [13].

2.7. SiRNA analysis

Small interference RNA (*SiRNA*) purification, separation and hybridization were performed essentially as described [20]. Twenty- and 23-mer oligonucleotides from *FAD*2 452 bp fragment were synthesized and used as molecular size markers and positive controls in hybridizations performed with DNA probe. Probes were the same as used in Southern blot. Prehybridization and hybridization were at 38 °C in the same solution to that used in southern blot.

3. Results

3.1. Characterization of cloned FAD2 cDNA

A putative tobacco microsomal ω-6 desaturase (*FAD*2) cDNA was cloned from tobacco leaf and the spliced whole sequence revealed an open reading frame of 383 amino acids (GenBank accession no. AY660024), which was 71–91% identical to *FAD*2 enzymes identified from some other plant species (data not shown).

The hydropathy plot of this putative *FAD*2 polypeptide was similar to its counterpart in *Abrabidopsis* described previously [7]. As shown in Fig. 1, it has four large hydrophobic regions that correspond to four membrane-spanning domains predicated in models of membrane-bound desaturases [21]. It also has three conserved histidine-rich boxes (His boxes), which may comprise the catalytic center of the enzyme; just as other desaturases do [5,22]. In the immediate vicinity of three His boxes, the Ala, Thr, Ser and Met residues were proposed to coordinate the two iron atoms at the active site center of the enzyme [23]. Furthermore, it contains a C-terminus aromatic amino acid-containing sequence (YKNKL) for maintaining localization in the ER [10]. In addition, it contains a motif consisting of Ser-Thr-Met, located just after the third His box, which is conserved among *FAD*2 enzymes [21].

3.2. Identification of primary transformants

Tobacco was transformed with gene-silencing constructs consisting of 452 bp fragment of *FAD*2 cDNA in inverted repeat configuration driven by a CaMV35S promoter (Fig. 2). The fragment downstream of 35S promoter was in sense orientation relative to CaMV35S. The neomycin phosphotransferase gene (*NPT*II) driven by the Nos promoter (Nos P) confers resistance to kanamycin, which served as selective marker for transgenic plant.

From kanamycin resistant calli, 63 seedlings were established, in which 37 lines showed a significant increase in oleic acid (18:1) content with a concomitant reduction of levels in linoleic (18:2) and linolenic (18:3) acids by analyses of fatty

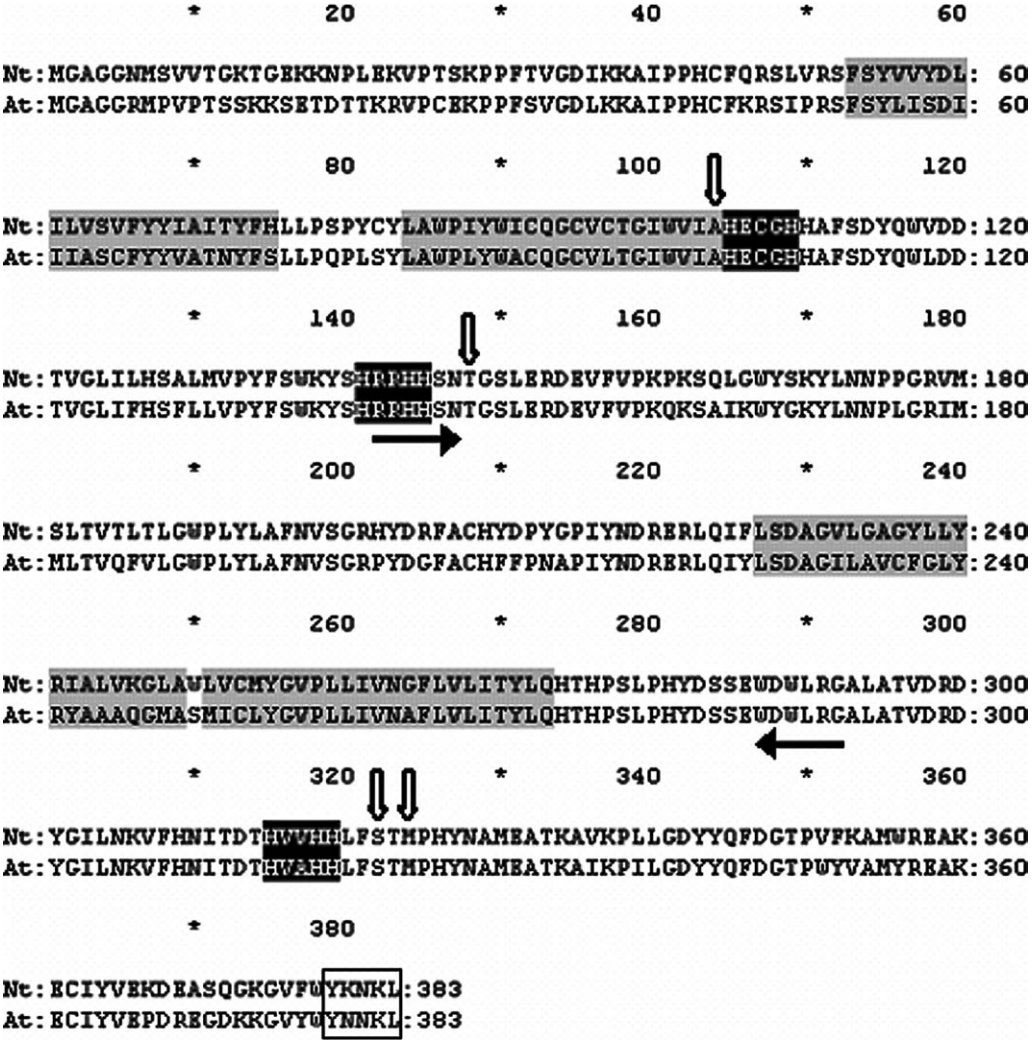

Fig. 1. Comparison of the *FAD*2 polypeptide sequences of *Nicotiana tabacum* (Nt) and *Arabidopsis thaliana* (At). The predicted membrane-spanning domains (gray) and highly conserved His boxes (black) are shown. Positions marked by open arrows are Ala, Thr, Ser and Met residues shown to be important determinants of *FAD*2 desaturase activity. The ER retrieval motif is boxed. The positions of the degenerated primers for the *FAD*2 452 bp fragment are marked by solid arrows.

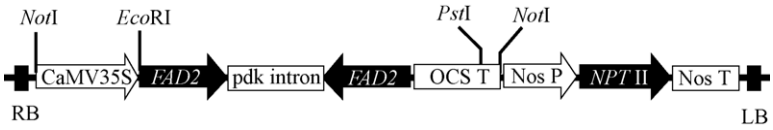

Fig. 2. Schematic diagram (not to scale) of *FAD*2-silencing construct used to transform tobacco. The construct driven by CaMV 35S promoter consists of an inverted repeat of the 452-nucleotide *FAD*2 coding sequence separated by intron of pdk [11]. The neomycin phosphotransferase selectable marker gene (*NPT*II) is driven by the Nos promoter (Nos P). The T-DNA left border (LB) and right border (RB) are shown. The positions of enzyme sites are indicated.

acid composition of total leaf lipids (Fig. 3). The regenerants can be visually grouped into two categories, one with average oleic acid content around 2% (similar to the controls) and another with high oleic content ranged from 8.3 to 16.3%, showing a marked increase up to eight-fold. However, the data showed that the transformation did not result in a morphological phenotype.

Southern blot analysis of genomic DNA was used to confirm the integration of the transgene into the tobacco genome and to estimate the transgene copy number. Genomic DNA prepared from the tobacco leaf tissue was digested with *Eco*RI and probed with [^{32}P]dCTP labeled partial coding sequence of *FAD*2 cDNA. As shown in Fig. 2, *Eco*RI cut once within the T-DNA of the *FAD*2-silencing constructs downstream of the 35S promoter. Three lines with high (S18 and S61) or medium (S28) level of 18:1 were selected as examples for southern blot analysis (Fig. 4). A 2.36 kb fragment from the *FAD*2-silencing construct digested with *Eco*RI and *Pst*I was used as positive control in hybridizations performed with *FAD*2 cDNA probe. As shown in Fig. 4, digestion of genomic DNA from wild-type and transgenic tobacco with *Eco*RI generates hybridizing fragments about 1.9 and 2.6 kb, respectively. Detection of two restriction fragments in tobacco genomic DNA is consistent with the existence of one *FAD*2 per genome of this allotetraploid plant. S18 contained single insertion while S28 and S61 contained several insertions provided that bands of different sizes absent in wild type are interpreted to represent different transgene insertions.

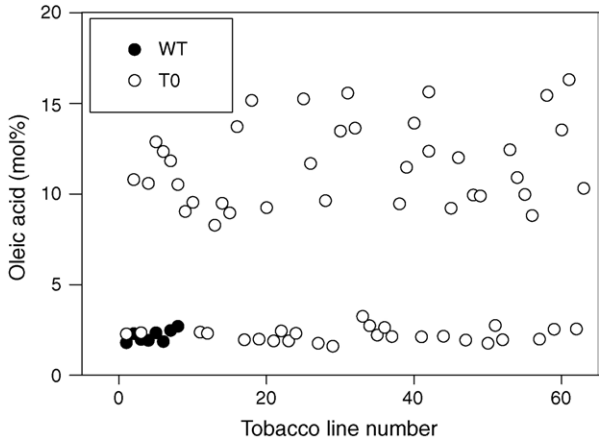

Fig. 3. Oleic acid contents of total lipids from tobacco leaves of seedlings grown at 24 °C. Wild-type seedlings (WT); seedlings established from kanamycin resistant calli (T0).

3.3. High oleic acid phenotype resulted from PTGS

A transgenic line (S61) with very high 18:1 levels was chosen to be representative to examine the *FAD*2 mRNA abundance in transgenic plants by Northern blot (Fig. 5A). Tobacco actin Tac9 gene (GenBank accession no. X69885) was used as internal control (Fig. 5B). Results showed that in S61, *FAD*2 mRNA dropped to a very low level in contrast with that of wild type. These results demonstrated a perfect correlation between the decrease of *FAD*2 mRNA and the increase of 18:1 in total leaf lipids. Therefore, we assume that the greatly elevated 18:1 content in transgenic plant leaves resulted from

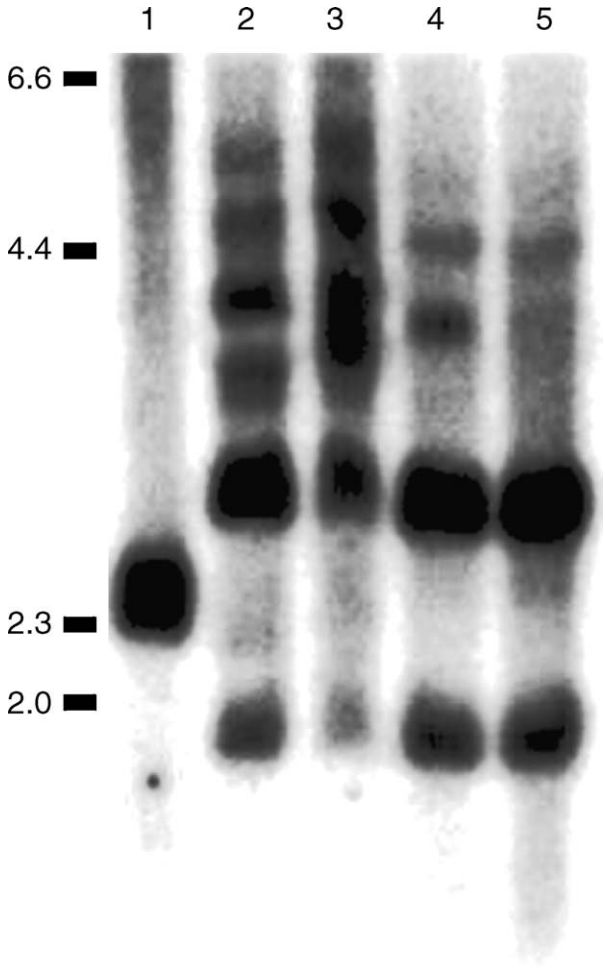

Fig. 4. Southern blot analysis of transgenic tobacco. Genomic DNA (20 μg) was digested with *Eco*RI, and the blot was probed with the [^{32}P]dCTP labeled 452 bp fragment of *FAD*2 cDNA. Lane 1, positive control; lane 2, S61; lane 3, S28; lane 4, S18; lane 5, wild type tobacco; DNA size markers are indicated on the left.

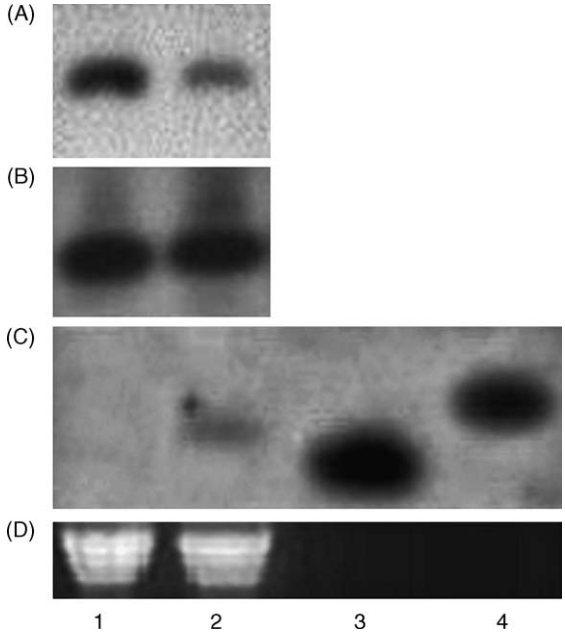

Fig. 5. Northern blot analysis of *FAD2* mRNA abundance (A) and *FAD2*-derived SiRNA accumulation (C) in leaves of S61. Fifteen microgram total RNA per lane for a, and 50 μg RNA with low molecular weight per lane for C. Both were hybridized with [^{32}P]dCTP labeled probes comprising the 452 bp coding sequence of *FAD2* gene. The blots for *FAD2* mRNA were stripped and reprobed with tobacco actin Tac9 gene as loading control (B). Ethidium bromide staining of low molecular weight RNA fraction is shown as loading control for SiRNA examination (D). Lane 1, wild type; lane 2, S61; lane 3, 20-mer oligonucleotide marker; lane 4, 23-mer oligonucleotide marker.

lower level of *FAD2* mRNA in transgenic lines induced by the silencing construct.

Low-weight-molecular RNA was extracted from leaves of S61 for Northern blot analysis to probe the presence of *FAD2*-derived small interfering RNA (SiRNA) to estimate if the low level of *FAD2* mRNA was caused by the silencing constructs. Analysis of RNA in the tobacco plants for the presence of *FAD2*-derived small (≈21 nt) RNA showed a perfect correlation between the presence of these molecules and the integration of the *FAD2*-silencing construct (Fig. 5C and D). The presence of *FAD2*-derived small RNAs (≈21 nt) in leaves of S61 can be interpreted as the *FAD2*-silencing construct was at work. This confirms that the reduced mRNA level and the increased 18:1 contents in transformants are due to PTGS, as such small RNAs are a hallmark of PTGS [20].

3.4. Effect of silencing constructs on individual polar lipids in leaves of S61

Individual lipids extracted from leaf tissues were subjected to fatty acid composition analysis for the silencing effect on specific polar lipids (Table 1). In the leaves of S61, both phosphatidylcholine (PC) and phosphatidylethanolamine (PE), the main lipid components of the extrachloroplastic membranes of plant cells, showed a marked increase in the level of 18:1 and a concomitant decrease in the amount of 18-carbon polyunsaturated fatty acid. The proportions of 18:1 increased to 56.4% in PC and to 28.7% in PE, respectively, which were more than five-fold over wild-type levels. Whereas those lipids (sulfoquinovosyldiacylglycerol, SQDG; phosphatidylglycerol, PG; digalactosyldiacylglycerol, DGDG; monogalactosyldiacylglycerol, MGDG) located predominantly in the chloroplast showed a relatively less increase in their 18:1 levels. It suggests that the silencing-constructs gave much more impact on fatty acid desaturation in extrachloroplastic membranes than in chloroplast, confirming its specific impact on the desaturation of oleic acid. Similarity between S61 and *Arabidopsis FAD2* mutant [24] in the profile of fatty acid composition suggests that tobacco *FAD2* gene was selectively silenced by the *FAD2*-silencing constructs. Thus, the tobacco *FAD2* gene was functionally identified since the most significant increase of 18:1 was found in PC of all individual lipids, with 18:2 and 18:3 decreased accordingly. In addition, these analyses suggest that the transgenic plants are deficient in the activity of a microsomal oleoyl-PC desaturase.

In individual polar lipids from *FAD2*-silenced leaves (Table 1), besides great variation of C18 unsaturated fatty acid, the increase of 18:1 was accompanied by a palpable reduction in the level of palmitic acid (16:0) in some individual polar lipids, as compared with wild type. The most significant reduction of 16:0 was found in PC and PE with a decrease of 38 and 30%, respectively.

3.5. Effect of silencing constructs on 18:1 content in different organs of S61

To determine whether the silencing constructs driven by CaMV 35S, a constitutive promoter, have the same effect on the different organs, analysis of fatty acid composition in different organs from S61 was carried out. Except for stems, the 18:1 levels in total lipids of all organs from S61 increased compared with that of wild type. The most significant elevation of 18:1 was found in leaves and seeds, whereas relatively less increase was found in roots, buds and flowers (Fig. 6).

The varied fluctuation of 18:1 levels in different organs may be caused by some deviation in strength of the activity of CaMV 35S promoter, driving the silencing construct, in different organs. To test this, RT-PCR was performed to estimate the transcript abundance in different organs of wild type and transformant (Fig. 7A and B). It showed that, in contrast to wild type, *FAD2* transcripts in S61 were much lower than that in all organs except for flowers. It suggested that the silencing constructs were at work and the CaMV 35S promoter probably had similar strength in these organs, at least in roots, stems, leaves and seeds. The varied fluctuation of 18:1 levels in different organs was therefore not due to different decrease of *FAD2* transcripts or any deviation in strength of the activity of CaMV 35S promoter in those organs of S61.

An oleic desaturation proportion (ODP) parameter can be used to assess the cumulative effect of *FAD2* activity [13]. Herein, we used ODP to estimate *FAD2* activity in different organs of tobacco. In wild-type plants, the ODP value was ranged from 0.64 to 0.73 for roots, stems, buds and flowers, and 0.91 and 0.98 for seeds and leaves, respectively, suggesting the constitutive *FAD2* activity in leaves and seeds were much

Table 1
Fatty acid composition of individual lipids from leaves of S61 (S) and wild-type (W) tobacco seedlings

Polar lipid	Fatty acid composition (mol%)							
	16:0	16:1	16:2	16:3	18:0	18:1	18:2	18:3
PC								
W	15.7 ± 0.4	–	–	–	5.5 ± 0.2	10.5 ± 0.3	42.9 ± 1.2	25.4 ± 1.2
S	9.8 ± 0.2	–	–	–	2.9 ± 0.2	56.4 ± 0.8	14.2 ± 0.6	16.7 ± 1.3
PE								
W	16.9 ± 0.3	–	–	–	4.4 ± 0.3	4.1 ± 0.3	40.8 ± 1.3	33.8 ± 1.6
S	11.9 ± 0.2	–	–	–	2.3 ± 0.4	28.7 ± 0.7	23.8 ± 0.8	33.3 ± 0.7
SQ								
W	39.3 ± 0.2	–	–	–	2.9 ± 0.2	4.4 ± 0.5	15.2 ± 0.5	38.2 ± 1.8
S	35.5 ± 0.3	–	–	–	2.9 ± 0.1	15.5 ± 1.0	14.6 ± 0.7	31.5 ± 1.4
PG								
W	31.7 ± 0.1	23.5 ± 0.2	–	–	1.9 ± 0.1	5.6 ± 0.6	14.8 ± 0.6	22.5 ± 1.5
S	29.1 ± 0.3	21.2 ± 0.1	–	–	2.2 ± 0.0	13.9 ± 0.5	11.3 ± 0.2	22.3 ± 0.6
DGDG								
W	11.8 ± 0.1	–	1.0 ± 0.1	1.3 ± 0.1	1.8 ± 0.0	1.7 ± 0.3	8.4 ± 0.2	74.0 ± 0.9
S	9.3 ± 0.2	–	1.0 ± 0.0	1.2 ± 0.1	1.1 ± 0.0	6.8 ± 0.6	6.8 ± 0.3	73.8 ± 1.4
MGDG								
W	3.0 ± 0.1	–	3.3 ± 0.1	11.9 ± 0.5	–	1.6 ± 0.2	9.5 ± 0.4	70.7 ± 1.6
S	2.1 ± 0.0	–	3.2 ± 0.2	12.1 ± 0.4	–	6.5 ± 0.2	8.8 ± 0.1	67.3 ± 1.8

Values are means ± S.D. ($n = 3$). Dashes indicate trace amounts (<1.0%). The 16:1 is Δ^3-trans-hexadecenoic acid. Replications are replicate isolations from the same plant.

higher than that of other organs. Because 18:1 content increased significantly in S61, ODP value in this line was much lower than that in wild type. However, the reduction extent of ODP value in this plant depended upon the organs. Except for stems, the decreases of ODP values in other organs of S61 were significant ($P < 0.01$), and the most reduction occurred in seeds and leaves in which the ODP value decreased ranging from 15 to 18%, which was far greater than 10% or even less represented in other organs.

4. Discussion

The features identified from the cloned cDNA, in particular, three histidine-cluster motifs containing eight histidine residues known to comprise the consensus sequence of fatty acid desaturases, are conserved in the putative *FAD*2 in tobacco. Furthermore, the analysis of individual lipids from transgenic tobacco showed that the most significant increase of 18:1 was found in PC, in which 18-carbon polyunsaturated fatty acids decreased accordingly. These results clearly demonstrate that the cloned gene encode a tobacco microsomal ω-6 desaturase. In addition, the result of RT-PCR demonstrated a constitutive expression of *FAD*2 in all tissues examined. In this work, a partially cloned cDNA of tobacco *FAD*2 was used to make a *FAD*2-silencing construct introduced into plant genome. The profile of fatty acid composition in S61 was comparable to that in *Arabidopsis FAD*2 mutant [24], which suggests that the tobacco *FAD*2 gene has been selectively silenced by the *FAD*2-silencing constructs.

The individual lipids analysis (Table 1) showed that, in addition to a remarkable increase of 18:1 in PC and PE, the 18:1

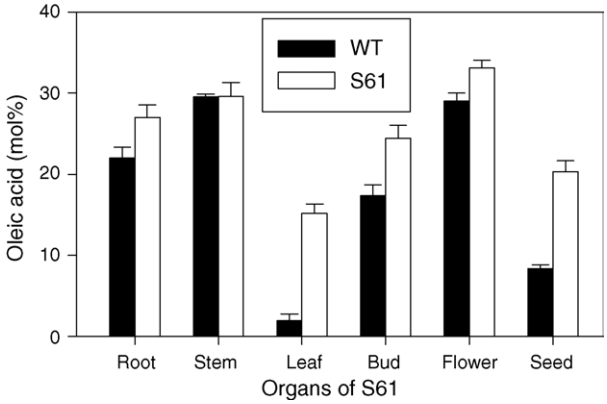

Fig. 6. Oleic acid contents in total lipids extracted from different organs of S61 and wild-type plants. Data of roots, stems and leaves were obtained from tobacco seedlings. Flowers were denuded of calyxes. Seeds were harvested at 28 day after flower (DAF). Values are means ± S.D. ($n = 3$).

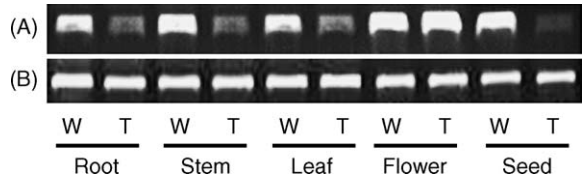

Fig. 7. RT-PCR analysis for determination *FAD*2 mRNA levels from different organs of S61 and wild-type plants. Ethidium bromide-stained gel of products obtained after RT-PCR analysis of mRNA from wild-type tobacco (W) and S61 (T) are shown (A). Control reactions were carried out using primers for tobacco actin Tac9 mRNA from the same samples (B). Data of roots, stems and leaves were obtained from tobacco seedlings. Flowers were denuded of calyxes. Seeds were harvested at 7 day after flower (DAF). Same results were obtained from two independent experiments.

levels in plastidial lipids (SQDG, DGDG and MGDG) also significantly increased, which caused a notable decrease in lipid ODP values. In plant cells, the 18:1 could be desaturated by FAD6 enzyme located in plastid as well as *FAD*2 [2]. The significantly increased 18:1 levels in plastidial lipids may be owing to cross silencing of FAD6 by the *FAD*2-silencing construct. However, both RT-PCR and SiRNA analysis demonstrate that the greatly elevated oleic acid content in leaves of transgenic plant is due to the reduction in *FAD*2 mRNA resulted from PTGS. The PTGS is a sequence-specific RNA degradation; therefore, the sequence of *FAD*6 and *FAD*2 should be sufficiently divergent that *FAD*6 could escape cross silencing by the *FAD*2-silencing construct used in the present study. Based on such observation, we suggest that, in *FAD*2-silenced transformants, 18:1 containing lipids in extrachloroplast may enter the chloroplast where they could be converted to 18:2 and subsequently to 18:3 by the plasitidial desaturases encoded by the *FAD*6, *FAD*7 and *FAD*8 genes. The resulted lipids with polyunsaturated fatty acids either remain in the plastid or are exported into protoplasm to be used for synthesis of the extrachloroplast lipids as suggested before [4]. Therefore, 18:2 containing diacylglycerol may not be the sole lipid molecule entering chloroplasts as described previously [8].

In *FAD*2-silenced tobacco, the 16:0 levels showed a significant reduction in some individual polar lipids, as well as marked fluctuation of C18 polyunsaturated fatty acids (Table 1). These phenomena were also found in *Arabidopsis FAD*2 mutant [24] and in *FAD*2-silenced plants of *Gossypium hirsutum* [12]. We assume that the *FAD*2-silencing may have effect on 16:0 synthesis de novo, since 16:0 in total lipids of leaves, seeds (data not shown) and in all the individual lipids declined to some extent. It has been suggested that the high degree of polyunsaturation may be required to maintain the degree of membrane fluidity in chloroplast or mitochondrial membranes [25,26] and for membrane function or membrane biogenesis at low temperature [27]. The marked increase of 18:1 with the concomitant decrease of 18:2 and 18:3 may affect the fluidity of membranes, which resulted in down regulation of 16:0 contents by unknown mechanism, to mitigate the change of membrane fluidity. The content of 16:0 in PG of plants was regulated by the culture temperatures have been reported previously [19]. In S61, the levels of 16:0 and Δ^3-trans-hexadecenoic acid, two high melting-point molecular species, decreased from 55.2% down to 50.3%, which may be a kind of adaptive response of *FAD*2-silenced plants under 24 °C used in this paper. The feature of lower level of 16:0 in *FAD*2-silencing plants than that in wild type make it possible to cultivate oilseed crops both rich in oleic acid and poor in palmitic acid.

Analysis of fatty acid composition of total lipids in different organs from *FAD*2-silenced plants (Fig. 6) suggested that the silencing constructs driven by a constitutive promoter have variant effect on the distinct organs. This phenomenon was also found in other *FAD*2-silenced lines, indicating it is not an anomaly for this particular transgenic line (data not shown). The result of RT-PCR (Fig. 7) demonstrated similar decrease of *FAD*2 transcripts in different organs except in flowers. This possibly because there are many haploid cells in flowers, especially pollen, which might not contain the silencing constructs owing to meiosis. The discrepancy between similar decrease of *FAD*2 transcripts and variant fluctuation of oleic acid in different organs of transformant might be explained by the existence of *FAD*2 isoenzyme (s) in ER of tobacco, just like other polyploid plants with two or more *FAD*2 isoenzymes in ER [28–30]. In these cases, the expression of one functional isoenzyme appears to be sufficient for the normal-oleate phenotype, but high-oleate phenotype may be established from manipulation of all isoenzymes [31]. The silencing construct used in this report, which is sequence specific, cannot silence its isoenzymes. In addition, the transformants showed less suppression of *FAD*2 than in *Brassica juncea* by antisense method, when assessed by the decrease of ODP value [32]. The silencing phenotype is so ''leaky'' may indicate there exists another *FAD*2 gene in tobacco. To sum up, the lack of correspondence between *FAD*2 transcript level and 18:1 content can be explained by differential expression of a second *FAD*2 gene, which was not picked up by the probe due to sequence differences.

The data (Figs. 6 and 7) clearly showed that the 18:1 levels in some organs had no direct relation to the abundance of *FAD*2 transcripts. In addition, ODP values derived from different organs of tobacco demonstrated variant constitutive activities of *FAD*2 in those organs, and the silencing construct showed severe effect on *FAD*2 in organs with high *FAD*2 activities than that in those organs with low. It has been shown that, in *Arabidopsis*, the level of *FAD*2 transcripts is several times in excess compared to the amount required for linoleate synthesis [7]. Another possible explanation for the leaky silencing phenotype is therefore the presence of detectable amounts of *FAD*2 transcripts in transgenic tobacco lines as shown in Figs. 5 and 7. A low level of *FAD*2 transcripts that survived the silencing machinery in *FAD*2-silenced tobacco was sufficient to allow a significant desaturase activity. Therefore, it is reasonable to assume that in some organs, such as roots and stems, a trace of transcript remains were enough for maintaining low *FAD*2 activity, which could not be down regulated by degradation of *FAD*2 transcripts; whereas other organs, such as leaves and seeds with high constitutive activity of *FAD*2, may need many transcripts for high *FAD*2 activity, which could be down regulated by degradation of *FAD*2 transcripts. Alternatively, owing to movement of lipid between chloroplast and ER is bi-directional [4], the flux of lipids from plastid to ER in some tissues is high enough to compensate for the loss of *FAD*2, and these need further investigation.

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