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REGULAR ARTICLE

Ectopic overexpression of *MucACP-Δ9* desaturase leads to ω-7 fatty acid accumulation in tobacco leaves

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ABSTRACT

MucACP-Δ9D from cat's claw (*Macfadyena unguis-cati*) was ectopically overexpressed in tobacco (*Nicotiana tabacum* L.) leaves to increase the content of omega-7 (ω-7) fatty acids, which have nutraceutical and industrial values for polyethylene and biofuel production. Overexpressed *MucACP-Δ9D* localized in the plastid resulted in an increased ω-7 fatty acid (C16:1Δ9 and C18:1Δ11) content from trace level in the wild type to 25.4-29.7% in the transgenic tobacco leaves. The C16:0, C18:2, and C18:3 fatty acid content decreased by 30-50%, 24%, and 38.7% in the transgenics compared with the wild type, respectively. This evidences that the ectopically expressed *MucACP*-Δ9D enzyme actively converts C16:0 to C16:1Δ9. *MucACP-Δ9D* overexpression in tobacco had no adverse effects on any agronomic traits, including plant growth, development, or seed germination. The present findings provide an excellent germplasm resource and a new technological path for commercial production of valuable fatty acids, using high-biomass vegetative organs of a non-food crop as platforms.

Keywords: Metabolic engineering; MucACP-Δ9 desaturase; Omega-7 fatty acid; Plant oil; Tobacco (*Nicotiana tabacum* L.)

INTRODUCTION

Omega-7 (ω-7) fatty acids, consisting of primarily palmitoleic acid (C16:1Δ9) and its elongation product, *cis*-vaccenic acid (C18:1Δ11), have unique features and are widely used in food, health care, as well as in the industry (Mozaffarian et al., 2010; Wu et al., 2011). Vegetable oils rich in such monounsaturated fatty acids can serve as ideal renewable sources for production of high-quality biodiesel in terms of stability, flammability, and cold tolerance (Wu et al., 2012). Conventional oil crops have a very low content of these valuable fatty acids (e.g. <1% in soybean seed). However, these unusual fatty acids can be synthesized in large amounts in several non-agronomic plants. For example, ω-7 fatty acid levels may reach up to 79%, 70%, and 32% in seeds of cat's claw (*Macfadyena unguis-cati*), *Kermadecia sinuate*, and sea buckthorn (*Hippophae rhamnoides*), respectively. However, due to low seed yield and poor agronomic traits, these naturally high accumulators have a limited commercial use for ω-7 fatty acid production. Consequently, an increasing biotechnological effort is aimed at modifying the lipid metabolic pathway in existing oilseeds for generating vegetable oils enriched in these highly valued fatty acids.

In plant cells, the key enzyme acyl-Δ9 desaturase (acyl-Δ9D) catalyzes the palmitic acid (C16:0) to form palmitoleic acid (C16:1Δ9). Although the C16:0 substrate of the acyl-Δ9D can be formed in conventional oil crops, only a tiny amount of C16:1Δ9 is produced because acyl-Δ9D has very low selectivity for C16:0 substrate in common oilseeds (Wu et al., 2011). Acyl-Δ9D can be divided into two classes based on their subcellular localization. These are plastidial acyl-ACP-Δ9 desaturase (ACP-Δ9D) and endoplasmic reticulum-localized cytosolic acyl-CoA-Δ9 desaturase (CoA-Δ9D). In some plants, such as cat's claw, the *de novo* synthesis of C16:1Δ9 occurs mainly in the plastid. Consequently, an increasing biotechnological effort is aimed at modifying the lipid metabolic pathway in existing oilseeds by metabolic engineering to generate vegetable oils enriched in these highly valued fatty acids (Nguyen et al., 2015; Haslam et al., 2016).

Unlike the conventional way to seed-specifically express C16:0-Δ9 desaturase in oil crops, this study uses high-biomass tobacco as the host to overexpress a cat's claw cDNA clone (*MucACP-Δ9D*) that encodes the plastidial C16:0-ACP-Δ9 desaturase in vegetative organs. In doing

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so, we aim to engineer a non-food tobacco for ω-7 fatty acid commercial production, particularly used for high-quality biodiesel. We predicted that the highest ω-7 fatty acid accumulation would be found in the best transgenic lines.

MATERIALS AND METHODS

Biomaterial

Tobacco (*Nicotiana tabacum* L. '*Samsun*' NN), is kept in our laboratory. Seeds were surface sterilized with 70% ethanol for 30 s, washed three times in distilled water (for 2 min each), followed by 0.1% $HgCl_2$ for 10 min and four washes in distilled water. The seeds were then plated on 1/2 strength Murashige and Skoog solid medium for germination in a growth chamber. Seedlings were grown in a greenhouse under a 16 h light (25 °C)/8 h dark (21 °C) photoperiod.

Experimental methods

MucACP-Δ9D expression vector construction

The complete coding sequence of the cat's claw plastid *acyl-ACP-⊿9D* gene, *MucACP-⊿9D* (GenBank accession no. AF051134), was amplified from a cloning vector kept in our laboratory. This MucACP-Δ9D showed a strong specificity to the C16:0-ACP substrate identified previously (Wu et al., 2012). Gene-specific primers with added restriction sites were designed (MucΔ9D-F1: 5'-CGAGCTCGATG GCCTTGAAGCTG-3' (underlined sequence: *Sac*I restriction site) and MucΔ9D-R1: 5'-GCTCTAG AGCTCA GAGTTG CACCTCTCT-3' (underlined sequence: *Xba*I restriction site)) and a high-fidelity PCR kit (New England Biolabs) was used to amplify the *MucACP-⊿9D* gene. Fig. 1 shows a schematic visualization of the cloning process of *MucACP-⊿9D* into the pCAMBIA1301 vector to construct the *35S²-MucACP-⊿9D* expression vector.

Tobacco transformation, reproduction of transgenic plants, and molecular identification

The *35S²-MucACP-⊿9D* expression vector was transformed into leaf discs of 8-week-old sterile tobacco seedlings using *Agrobacterium*-mediated transformation. For the selection, 5 mg/l of hygromycine (HPT) was used. PCR and Southern blotting were applied to detect the presence of *MucACP-⊿9D* gene in the T_0 transformed tobacco samples. Southern blotting was conducted to detect target gene integration according to the method described by Vanhercke et al. (2014). The seeds harvested from T_1 transgenic plants were placed on solid medium containing the antibiotic for germination. Positive seedlings were then transferred to soil plots for growing until maturation.

qRT-PCR was used to detect the expression of *MucACP-⊿9D* gene in T_2 transgenic plants. As a reference gene, we used 18S RNA. The qRT-PCR reaction program was designed as: 94 °C for 5 min, followed by 30 cycles of 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s. A dissociation curve was performed after the PCR cycle to confirm that only one PCR product was amplified. The expression level

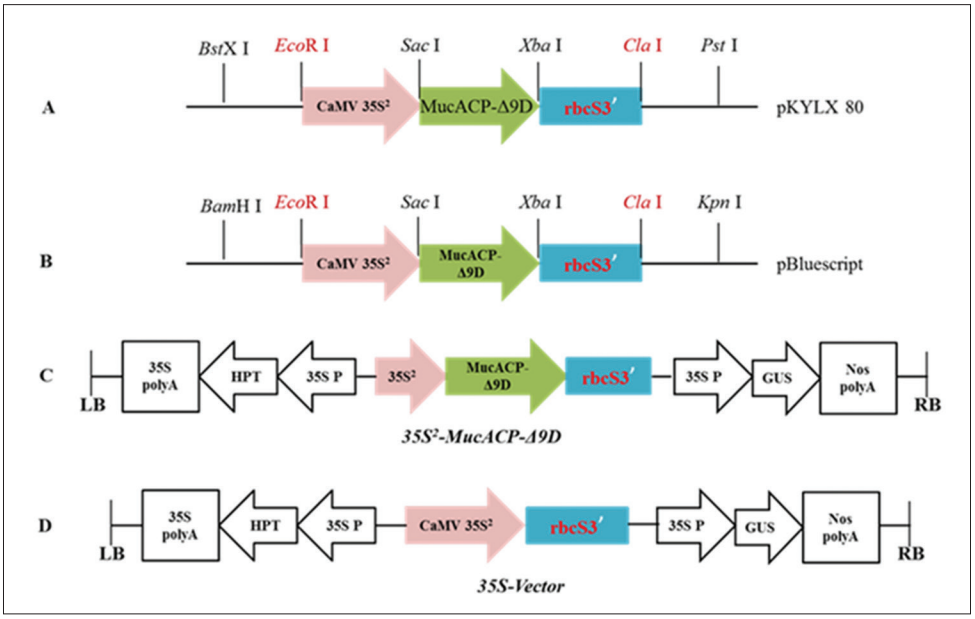

Fig 1. Schematic representation of *MucACP-Δ9D* expression used for tobacco transformation. A. *MucACP-Δ9D* was sub-cloned into vector pKYLX 80; B. *MucACP-Δ9D* expression cassette was inserted into the pBluescript intermediate vector; C. *MucACP-Δ9D* expression cassette was inserted into the plant binary expression vector pCAMBIA 1301 (*35S²-MucACP-Δ9D*); D. The expression vector without the *MucACP-Δ9D* gene (35S-vector) served as the empty vector control. P: Promoter. HPT: Hygromycin resistance gene. 35S polyA: 35S poly (A) signal sequence. Nos polyA: Nopaline synthase poly (A) signal sequence.

of the *MucACP-Δ9D* gene in different qRT-PCR samples was calculated based on the $2^{-\Delta\Delta CT}$ method as described (Livak and Schmittgen, 2001).

Detection of subcellular localization of MucACP-Δ9D enzyme in transgenic tobacco

Bioinformatics analysis showed that the *MucACP-Δ9D* sequence contained a plastid signal sequence section. In addition, the enzyme localized to the chloroplast of the cat's claw cells (Cahoon et al., 1998). In order to verify whether the MucACP-Δ9D was localized in the plastid, the subcellular localization of the enzyme was assessed by western blotting. For construction of a subcellular-targeting expression vector, six His-tag sequences with enzyme sites were inserted at the C terminus of MucACP-Δ9D by adding the His-tag sequences to the 3'-primer for amplifying the *MucACP-Δ9D*.

Proteins from chloroplast, microsome, nucleus, and whole cells of leaf samples were isolated with a sucrose density gradient centrifugation as previously described (Roughan et al., 1980). Western blotting was performed using standard procedures, 35 mg of sample was obtained and used in a 12.5% SDS-PAGE separation (Mini-Protein II System, Bio-Rad). The separated proteins were transferred to a nitrocellulose membrane (semi-dry transfer blotter, Bio-Rad) in TBST (TBS + 0.05% Tween20) electroporation buffer. The nitrocellulose membrane that contained the transferred proteins was blocked and incubated in 3% BSA-TBS buffer for 1 h. Then, the membrane was incubated with His-tag monoclonal antibody (1:2000 dilution) for 3 h. The blotting membrane was then washed in TBST and incubated with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG) to detect the primary antibodies bound to the MucACP-Δ9D protein by electrochemiluminescence technology according to the manufacturer's protocol (Amersham Biosciences).

Fatty acid extraction and GC analysis of the transgenic MucACP-Δ9D-expressing tobacco

To investigate the impact of *MucACP-Δ9D* overexpression on fatty acid composition, 6-week old transgenic plants were selected for the extraction of total fatty acid as described in Dahmer et al. (1989). Briefly, about 10-20 mg of freeze-dried leaf sample was placed in a glass test tube that contained 3 ml of a 2% (v/v) sulfuric acid/methanol. Triheptadecanoin (C17:0) was added as an internal standard (10 μg/10 mg sample). The mixture was thoroughly ground and then treated at 80 °C for 10 min to produce a 0.4 ml liquid sample. Next, 1 ml of 0.01% (w/v) butylated hydroxytoluene in hexane was added and the mixture was centrifuged a few minutes. The upper phase (the hexane phase contains the fatty acid methyl esters, FAME) was transferred to the gas chromatography (GC) system (0.25 mm i.d. × 0.33 μm × 10 m FFAP column, flame ionization detector, Agilent 7890A) for the detection of various fatty acids. The sample was incubated at 120 °C for 1 min. The temperature was increased by 12 °C/min to 210 °C, held for 3 min, followed by another increase by 5 °C/min to 235 °C, held for 8 min. The injector and detector temperatures were 220 °C and 250 °C, respectively. The carrier gas was helium with a flow rate of 10 ml/min.

RESULTS

The identification of transgenic *MucACP-Δ9D* tobacco lines

A total of 124 hygromycin-resistant transgenic tobacco plants from independent disc transformations were obtained in the T_0 generation. From these plants, 73 transgenic lines expressing *MucACP-Δ9D* were obtained in the T_2 generation as detected by PCR and qRT-PCR. The qRT-PCR results from leaf samples indicated no statistically significant ($P > 0.05$) variation in *MucACP-Δ9D* expression between the different transgenic lines. Twenty expression lines of *MucACP-Δ9D* from independent transformation events were selected for subsequent analysis.

Subcellular localization of the *MucACP-Δ9D* enzyme protein in transgenic tobacco

Western blotting showed that the enzyme was present in chloroplast and cellular protein in the *MucACP-Δ9D* overexpressing lines (Fig. 2). By contrast, MucACP-Δ9D was not detected in the microsome, nuclear protein fractions, or in the control group. This is consistent with MucACP-Δ9D sequence analysis, which showed that MucACP-Δ9D enzyme could enter host chloroplasts under the guidance

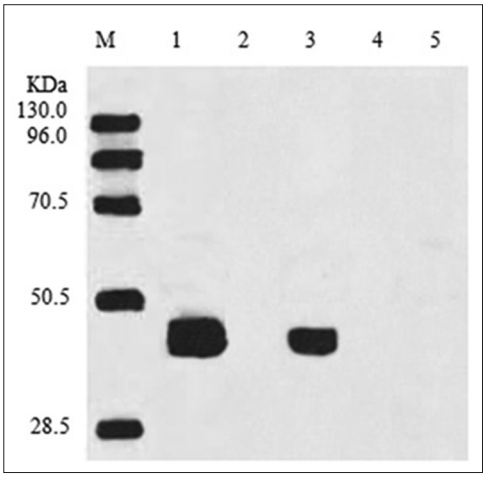

Fig 2. Subcellular localization of MucACP-Δ9D in transgenic tobacco. A representative western blot of transgenic tobacco indicating the discrete localization of MucACP-Δ9D in the chloroplast. M: protein marker. 1-4: MucACP-Δ9D from overexpressing transgenic plants, 1: total cellular protein, 2: nuclear protein, 3: chloroplast protein, 4: micro-protein, 5: total protein of the empty vector.

of a signal peptide. Therefore, this foreign enzyme was correctly localized in the chloroplasts, as expected.

Fatty acid composition analysis of transgenic tobacco leaves expressing *MucACP-Δ9D*

GC analysis showed that the most significant changes in fatty acid composition were observed in transgenic leaves compared to the untransformed tobacco (Fig. 3). In particular, transgenic germplasms overexpressing *MucACP-Δ9D*, showed a drop in C16:0 content from 26% in the control group to 13.4-18.3%. In contrast, the C16:1Δ9 content was very low in the control group (<0.4%) but was significantly increased to 16.4-20.4% in the transgenic lines. Further statistical analysis showed that the C16:0 and C16:1Δ9 content were negatively correlated (r = -0.946**) (Table 1). These results indicate that in the transgenic lines, this enzyme catalyzed the conversion of ACP-16:0 to ACP-16:1Δ9 with relatively high catalytic activity.

The change in 18C fatty acid content showed a trend similar to that observed in 16C fatty acid in the *MucACP-Δ9D*-overexpressing tobacco lines (Fig. 3). Most notably, the significant increase in C18:1Δ11 synthesis and accumulation in transgenic plants reached an average content of 9.0%, but was very low in the control groups (empty vector and non-transgenic plants). In addition, the changes in C18:1Δ11 and C16:1Δ9 content exhibited a similar uptrend. Statistical analysis showed a positive correlation between the content of C18:1Δ11 and C16:1Δ9 (r = 0.864**), and a negative correlation with the C16:0 content (r = -0.71**) (Table 1). The correlation suggests that in transgenic lines, the MucACP-Δ9D enzyme catalyzed C16:0 to produce C16:1Δ9, which further elongated to C18:1Δ11. Overall, when *MucACP-Δ9D* is overexpressed in tobacco leaves, the ω-7 fatty acid (C16:1Δ9 and C18:1Δ11) content increased from trace level in the control to 25.4-29.7% in transgenic lines. These results indicate that a significant production and accumulation of ω-7 fatty acids occur in the transgenic lines.

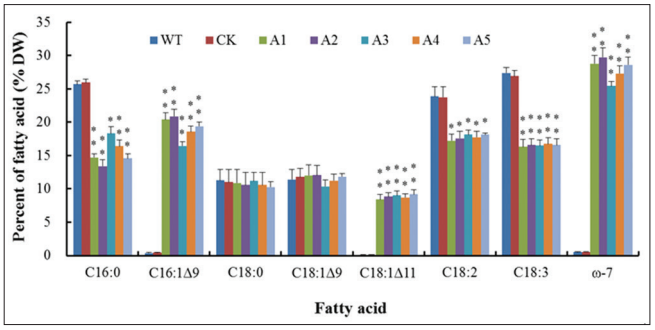

Fig 3. Fatty acid content in the *MucACP-Δ9D*-overexpressing tobacco leaves. WT: Non-transformed wild type leaves. CK: Empty vector infected leaves. A1-A5: Transgenic *MucACP-Δ9D* tobacco lines. Statistical significance was set to $^{*}P < 0.05$ and $^{**}P < 0.01$.

Unlike the reduction of C16:0 fatty acids, C18:0 did not show a significant change in the transgenic lines. However, two other 18C polyunsaturated fatty acids, linoleic acid (C18:2) and linolenic acid (C18:3), were significantly decreased in transgenic tobacco leaves. The C18:2 content dropped from an average of 23.3% in the controls to 17.7% in the transgenic lines. The C18:3 content dropped from an average of 26.9% in the control group to 16.5% in the engineered lines. Statistical analysis indicated that changes in C18:2 and C18:3 polyunsaturated fatty acids were positively correlated with changes in C16:0 fatty acid, and negatively correlated with changes in C16:1Δ9 and C18:1Δ11 (Table 1).

Impact of *MucACP-Δ9D* overexpression on the tobacco phenotype

Although relatively large changes in the fatty acid content were detected in *MucACP-Δ9D*-overexpressing transgenic tobacco leaves, the fatty acid composition in tobacco seeds did not change significantly (data not shown). In addition, no significant changes were observed in plant morphology (Fig. 4), seed germination rate (Fig. 5), or leaf chlorophyll content (Fig. 6) compared to the control plants. These results indicate that *MucACP-Δ9D* overexpression significantly improved the fatty acid composition in tobacco leaves without any adverse effects on the growth and development of the plants.

The desaturation effect of *MucACP-Δ9D*

In order to investigate the catalytic efficiency of MucACP-Δ9D in the tobacco plastid, we calculated the C16:0 index of desaturation (C16:1Δ9/(C16:0 + C16:1Δ9) ×

Table 1: Correlation coefficient (r) between the proportion of fatty acids in the *MucACP-Δ9D*-overexpressing transgenic tobacco leaves (n=31)

Fatty acid	C16:0	C16:1Δ9	C18:0	C18:1Δ9	C18:1Δ11	C18:2	C18:3
C16:0	——						
C16:1Δ9	–0.946**	——					
C18:0	0.568*	–0.387*	——				
C18:1Δ9	–0.591*	0.339	–0.599*	——			
C18:1Δ11	–0.710**	0.864**	–0.230	–0.135	——		
C18:2	0.805**	–0.952**	0.136	–0.105	–0.889**	——	
C18:3	0.742**	–0.909**	0.144	0.066	–0.976**	0.960**	——
ω-7	–0.901**	0.988**	–0.350	–0.055	0.932**	–0.961**	–0.957**

Statistical significance was measured at $^{*}P<0.05$ and $^{**}P<0.01$.

100), which expresses how extensively C16:0 has been desaturated into C16:1Δ9. In comparison to the wild type plants, the desaturation index of C16:0 increased 30-40 times in the transgenic leaf samples. This showed an average increase of 36.9 times more than control plants (Table 2). These results indicate that MucACP-Δ9D had a very efficient desaturation effect on saturated fatty acids in transgenic tobacco leaves.

Fig 4. Phenotypic comparison of transgenic and wild type tobacco plants. *MucACP-Δ9D*-overexpressing tobacco plant (A) and a wild type plant (B).

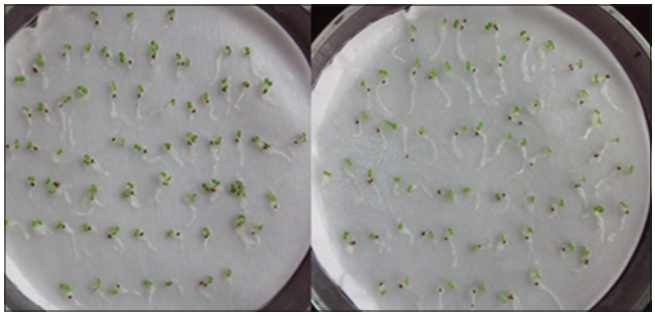

Fig 5. Germination of transgenic and wild type tobacco seedlings. Germination was not different between the *MucACP-Δ9D*-expressing (left) and wild type (right) tobacco seeds.

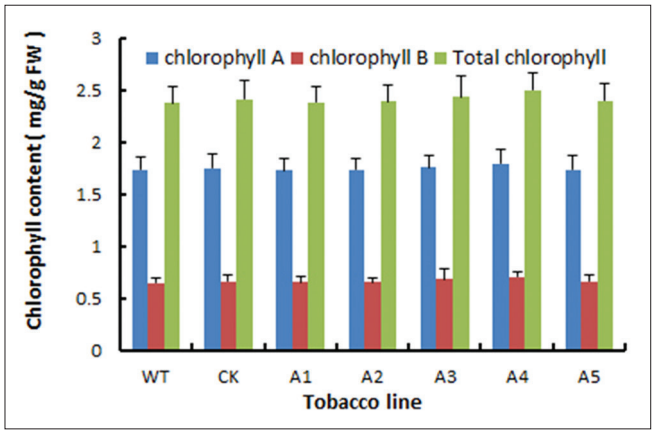

Fig 6. Leaf chlorophyll content of the *MucACP-Δ9D*-transgenic plants and control plants leaves. WT: non-transformed wild type tobacco. CK: empty vector infected tobacco. A1-A5: transgenic *MucACP-Δ9D* tobacco lines.

DISCUSSION

The application of metabolic engineering to improve special fatty acid composition in conventional oil crops is a new strategy developed in recent years. The aim of this strategy is to improve the stability, quality, and nutritional value of vegetable oils for the purpose of wider utilization. One strategy to enhance the synthesis of ω-7 fatty acids and improve their accumulation is to overexpress *ACP-Δ9D* and/or *CoA-Δ9D* in target plant seeds (Grayburn et al., 1992; Nguyen et al., 2010; Xue et al., 2013; Nguyen et al., 2015). Here, the *MucACP-Δ9D* gene was isolated from the ω-7 fatty acid-rich wild cat's claw and inserted into an overexpression vector for the transformation of tobacco plants. Transgenic tobacco lines with a high level of ω-7 fatty acid synthesis and accumulation were obtained. In *MucACP-Δ9D*-expressing transgenic tobacco lines, the content of these two types of ω-7 fatty acids increased by 25.4-30.0% compared to wild type tobacco.

Different sources of ACP-Δ9D could have different selectivity for the substrates C16:0-ACP and C18:0-ACP (Wu et al., 2011). Transgenic *Arabidopsis* seeds that expressed the *Asclepias syriaca* ACP-Δ9D did not accumulate ω-7 fatty acid. However, in *Doxantha ACP-Δ9D*-expressing transgenic *Arabidopsis* seeds, newly synthesized ω-7 fatty acid was detected (Nguyen et al., 2010). In wild cat's claw, the ω-7 fatty acid composition of seed oil could be up to 79% (64% C16:1Δ9, 15% C18:1Δ11). This indicates a strong catalytic activity of this acyl-Δ9D for ω-7 fatty acid synthesis. The plastidial acyl-Δ9D in cat's claw has strong substrate specificity for C16:0-ACP (Cahoon et al., 1998), and also in the yeast cells of this *acyl-Δ9D* expression (Yue and Li, 2009). Our work with transgenic tobacco further confirms the substrate specificity of cat's claw acyl-Δ9D to C16:0-ACP.

Table 2: Index of C16:0-desaturation in *MucACP-Δ9D* transgenic and control tobacco

Line	Index of C16:0 desaturation
WT	1.48
CK	1.52
A1	58.06
A2	60.89
A3	47.27
A4	53.14
A5	57.06

WT: non-transformed wild type leaves. CK: empty vector infected leaves. A1-A5: transgenic *MucACP-Δ9D* tobacco lines

In this study, transgenic *MucACP-Δ9D*-expressing tobacco leaves had increased levels of two ω-7 fatty acids: C16:1Δ9 and the extension product C18:1Δ11. However, another type of ω-7 fatty acid, the C20:1Δ13 was not detected. The C16:0 saturated fatty acid content was significantly decreased, but the saturated fatty acid C18:0 and monounsaturated oleic acid (C18:1Δ9) content did not change significantly in transgenic plants. The introduction of foreign *MucACP-Δ9D* cDNA into different plant species has led to an increase of the ω-7 fatty acids content in the seed oil; however, the rate of increase and the changes in other fatty acids vary significantly in different plants (Bondaruk et al., 2007). These results indicate that subtle differences might exist in the lipid biosynthesis and metabolic pathways among different plants. The differences could be due to different feedback mechanisms in the upstream or downstream synthesis and accumulation pathways of the target fatty acid. Therefore, simultaneous engineering of the upstream and downstream steps in the ω-7 fatty acid biosynthesis pathway would help to decrease the content of saturated fatty acids, and increase ω-7 fatty acid accumulation.

The *MucACP-Δ9D* gene contains a sequence of a signal peptide for plastid targeting. After translation, the signal sequence guides the MucACP-Δ9D enzyme into the chloroplast. Our data confirms that the heterologous MucACP-Δ9D enzyme functioned properly in the chloroplasts of transgenic tobacco cells. The expression of ACP-Δ9D in chloroplasts generally results in increased production of the target fatty acids. It is possible that C16:0, the precursor of C16:1Δ9, was synthesized in the plant chloroplast/plastid, facilitating enzyme access to the C16:0 substrate, and consequently, increased the catalytic efficiency of the acyl-ACP-Δ9D in the plastid. The present data showed that, following the increase of the monounsaturated fatty acids C16:1Δ9 and C18:1Δ11, the content of the polyunsaturated fatty acids C18:2 and C18:3 decreased correspondingly in the transgenics. This is consistent with previous results in which tobacco was transformed with rat acyl-Δ9D (Grayburn et al., 1992). Additionally, in *acyl-Δ9D*-expressing transgenic tobacco plants, as the monounsaturated fatty acid content increased, the saturated fatty acid content decreased. However, the average number of double bonds in each glycerol molecule was not significantly different between the control and transgenic plants (Moon et al., 2000). The specific mechanism of this phenomenon is still unclear, and it is possible that plant cells have evolved a fine adjustment mechanism that regulates the level of unsaturated fatty acids. Thus, the reduction of C18:2 and C18:3 polyunsaturated fatty acids in transgenic *MucACP-Δ9D* tobacco leaves could compensate for the increase in monounsaturated fatty acid content. The expression of foreign genes could have an impact on the metabolic network of the host cells and may generate some unexpected outcomes. Wang et al. (1996) transformed yeast acyl-Δ9D into tomatoes, and the transgenic fruit showed an increase in C16:1Δ9 and C18:2 content, whereas the content of oleic acid (C18:1Δ9) and C18:0 decreased. This may be caused by the different source of acyl-Δ9Ds and the host plants.

Finally, the analysis of fatty acid composition of transgenic tobacco seeds showed only a slight change that was not significantly different from the controls. Moreover, seed germination rate and other phenotypes of the transgenic plants were not significantly different compared to the wild type. Recently, the high-oil tobacco lines with leaves rich in oil (>10%) were successfully obtained by metabolic engineering (Vanhercke et al., 2014). In the future, such high-oil tobacco lines can be combined with ω-7 fatty acid-rich tobacco lines to develop novel tobacco germplasms for sustainable green production of the high-quality biofuels and other valued products.

CONCLUSIONS

We have substantially enhanced ω-7 fatty acid (C16:1Δ9 and C18:1Δ11) content in high-biomass tobacco leaves by ectopic overexpression of a cat's claw *MucACP-Δ9D*. Correspondingly, the saturated and polyunsaturated fatty acid (C18:2 and C18:3) content decreased. This illustrates the great potential of upregulating the biosynthesis pathways of specific fatty acids. The present findings provide a new technological path for commercial production of the valued fatty acids using the high-biomass vegetative organs of the non-food crop as platforms.

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Authors' Contributions

Chang-Yong Gao: Design, formulation of experiment with writing manuscript. Xue Mao: Collection of data. Hong-Qin Shang: Statistical analysis of data. Xia-Jie Ji: Transformation and identification of transgenic plants. Run-Zhi Li: Designed the study and corrected the article.

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