ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2021; 26(5): 2971-2978
doi: 10.25083/rbl/26.5/2971.2978

Received for publication, April, 26, 2021
Accepted, August, 9, 2021

Original paper

Gene Transformation by Chitosan Nanoparticle to Enhance Fatty Acid Production in Zea mays (L.)

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Abstract

Zea mays is an important crop and an essential source of fatty acids. Hence, increasing and adding new fatty acids led to the enhancement of these properties. Transformation of external Acetyl-CoA gene (Aco) can enhance fatty acid components, as ACo is expressed into Acetyl-CoA carboxylase (ACCase) enzyme, which is the first essential step in the fatty acid production process. Chitosan nanoparticles are safe and fast polymer nanoparticles that are applied for gene transformation. Conventional PCR was performed for the detection of the ACo gene in both transgenic and non-transgenic maize lines. The results confirm the presence of the gene in the transgenic lines and absence in non-transgenic lines. The Gas chromatography-mass spectrometry (GC-MS) analysis for fatty acid contents in transgenic and non-transgenic maize lines showed an increase in fatty acid contents in transgenic lines compared to non-transgenic ones. Besides, the transgenic maize's lines produced extra new fatty acids not found in non-transgenic ones.

Keywords Acetyl-CoA carboxylase; Chitosan Nanoparticles; Fatty Acids; (GC-MS); Gene transformation; *Zea mays*.

To cite this article: HUSSIEN ET, DAHAB MI, ABD-ELATTY KM, EL-SHENAWY IH, FAROUK HM, RIZK AT, KAMAL NA, ABD-ELWAHAB RA. Gene Transformation by Chitosan Nanoparticle to Enhance Fatty Acid Production in *Zea mays* (L.). *Rom Biotechnol Lett*. 2021; 26(5): 2971-2978. DOI: 10.25083/rbl/26.5/2971-2978.

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Introduction

Zea mays (L.), also known as corn, is a monocot cereal grain with a leafy stalk that produces ovuliferous inflorescences called ears that yield kernels or seeds, which are fruits. Maize is a species in the family Poaceae. The corn has a high hectare production, maize grains have a high nutritional value. Maize grains have a high nutritional value and a complex chemical composition. These values may vary depending on the biological material studied. Maize flour is nutritious, tasty, easy to digest, regularly consumed, helps cleanse the colon, helps digestion by reducing stomach acid. The chemical composition of maize (for 100 g) is as follow carbohydrate 71.88%, protein 8.84%, fat 4.57 g, fiber 2.15 g, ash 2.33 g, moisture 10.23 g, phosphor 348 mg, sodium 15 mg, Sulfur 114 mg, Riboflavin 0.10 mg, Amino acids 1.78 mg, and other different minerals with variable concentrations (Shah et al., 2015; Murdia et al., 2016; Ghețe et al., 2018).

Acetyl-CoA carboxylase (ACCase) is an expressed enzyme that catalyzes the first step of fatty acid formation and the carboxylation of acetyl-CoA to malonyl-CoA. Heteromeric ACCase consists of four subunits in prokaryotes, wherein eukaryotes it is homomeric ACCase and composed of a single large polypeptide. The ACCase level is at least transcriptionally and post-transcriptionally controlled during organism development. This enzyme catalyzes the first committed step of fatty acid synthesis, and not only control of the enzyme level but also control of its activity is vital for organism life (Konishi et al., 1996; Sasaki and Nagano, 2004).

The transformation of the gene of interest identified is by different methods classified into two types (indirect and direct). The indirect method involves the use of a vector to deliver the gene. The direct method is directly by various chemical and physical ways of gene transfer. The genetic diversity that exists in available species is analyzed to identify the gene of interest to develop an elite cultivar (Kavipriya et al., 2019). Chitosan nanoparticles provide an efficient and rapid direct method for gene transformation. An advantageous charge on the DNA conjugate nanomaterial surface can be identified by biodegradable polymers such as chitosan. Chitosan is a polymer that has been commonly used in packages of nucleic acid delivery and tissue engineering (Raftery et al., 2013; Li et al., 2015).

Chitosan-based polysaccharides have gained significant attention in recent years as new functional biomaterials with potential applications in various fields. Chitosan chemical properties are insoluble in most solvents but slightly soluble in dilute organic acids such as acetic, lactic, malic, formic, and succinic acids. The degree of deacetylation (DDA) of a chitosan biomaterial expresse the actual molarity of the glucosamine residue in the polymer chain. This indicates the cationic charge on the molecule once diluted in acid solution. This is evident from the proportion of free amino groups in the chitosan biopolymer (Shigemasa et al., 1996; Periayah et al., 2016). Chitosan-derived biomaterials have received considerable attention as an antimicrobial, functional, renewable, non-toxic, biocompatible, bioabsorbable, and biodegradable biopolymer agent (Seda et al., 2007; Zhang et al., 2010).

This study aims to design a new transgenic *Zea mays* line able to produce more fatty acids for biodiesel production. Acetyl-CoA is the target gene expressed into Acetyl-CoA carboxylase (ACCase) (enzyme that catalyzes the first step of fatty acid formation). This gene was transformed via chitosan nanoparticles.

Materials and methods

Obtaining of Acetyl-CoA and Preparation of modified plasmid

Acetyl-CoA gene, with accession number: X14825, was located in genome of *E. coli* (DH5-α). The total genomic DNA of *E. coli* was isolated using the CTAB method according to Edwards et al. (1991). The PCR reaction required 2µl of 50ng DNA for each 25µl reaction, 12.5µl of 2X master mix (Biolene), 1µl of each forward and reverse primer (50nmole/base) (Biosearch, #P 1-5) and complete up to 25µl by sterile ddH_2O. The primers were designed using snap gene® (2.3.3) software; ACC-F; 5'-GACCTCGTCCTCCCTGAC-3′, ACC-R: 5′- CCGCGGT TGGCAATAACAAT-3′. PCR program was repeated 40 cycles as follows: 30 sec at 95ºC, 30 sec at 50ºC, and 1 min at 72ºC. The PCR product was run on 1.4% (w/v) agarose gel comparing to (New England Biolab, #N3232S) ladder. The *ACC* product was ligated separately to pMiniT Vector (NEB® PCR Cloning Kit, #E1202S. The kit was supplied with NEB 10-beta Competent *E. coli* (NEB #C3019)) following the manual instruction.

Confirmation of Bacterial Transformation (Colony PCR)

This step was applied to recognize recombinant and non-recombinant colonies using colony PCR. Bacteria containing modified plasmids were grown on LB agar plates supplemented with 100 µg/L ampicillin. Single colonies were used as a template for PCR reaction using the same ACC primers then, check the product on 1.4% gel electrophoresis.

Chitosan nanoparticle Characterization

Infrared Spectroscopy (FTIR-ATR)

FTIR-ATR analysis was performed at wavelengths between 7500 and 360 cm^{-1} using a Bruker Optic GmbH (alpha model, Laser class 1) Spectrometer with attenuated total reflectance (Siafu, 2017).

X-Ray Diffraction.

The XRD analysis was carried out using Rigaku Corp. D/Max-2500/PC X-ray Diffractometer was equipped with a back monochromator operating at 40 kV and 100mA at the scanning range of 5°–80° with a step size of 0.1∘ and a time/step of 1 s using copper cathode (Cu Kα1) as the X-ray source (λ = 1.54056 ˚ A) (Siafu, 2017).

Scanning Electron Microscopy (SEM)

The samples surface morphology was investigated by using Field Emission Scanning Electron Microscopy (Hitachi S-4800, Japan). The accelerated voltage was 15 kV and according to the instruments and software protocol (Siafu, 2017).

Particle Size and Size Potential

Chitosan nanoparticle was characterized in term of size and zeta potential by dynamic light scattering (DLS) according to the instruments and software protocol using the Malvern Zeta-sizer Nano ZS (Malvern Instruments Inc., Southborough, MA) with Malvern (7.2) software.

Preparation of chitosan-DNA nanoparticles (CS/pDNA)

Mansouri et al. (2006) designed the protocol of CS/DNA formation as follows: chitosan nanoparticles (CS) were dissolved in 25mM acetic acid and adjusted to pH 5.5 at a concentration of 0.08%. Both CS and recombinant pMiniT were first incubated in a water bath at 55ºC for 15min and then added equally to each other with stirring on a vortex for 1min.

Transformation of Chitosan/pDNA into *Zea mays*

The method of chitosan nanoparticle transformation into plant tissue was developed by Abdel-Razik et al. (2017): Seedlings of *Zea mays* were inoculated by a syringe containing CS/pDNA complex, then incubated at 25±1°C for 2 weeks to regenerate transgenic plantlets.

Molecular analysis of Transgenic Zea mays Lines

Molecular analysis was performed for both transgenic and non-transgenic lines to confirm gene transformation to transgenic ones. DNA from both leaves of transgenic and non-transgenic *Zea mays* lines was isolated, then PCR was applied with the same *ACC* primers and the same PCR program conditions to confirm gene transformation.

Fatty Acids Analysis

The total lipids were extracted following the protocol of Madany and Khalil (2017) with some modifications. A mixture of Chloroform and methanol (1:2) was added to 5g of transgenic and non-transgenic maize to extract the total lipids. They are ground well to separate the organic phase containing the total lipids. Cell residue was removed by filtering through Whatman GF/C paper. Then the fractions were analyzed using Gas-Chromatography-Mass Spectroscopy (GC-MS).

GC-MS Analysis for Total Lipids

Mass spectra were recorded using Shimadzu GCMS-QP2010 (Koyoto, Japan) equipped with Rtx-5MS fused bonded column (30 m x 0.25 mm i.d. x 0.25 µm film thickness) (Restek, USA) equipped with a split–splitless injector. The initial column temperature was kept at 50°C for 3 min (isothermal) and programmed to 200 °C at a rate of 15 °C/min and kept constant at 200 °C for 5 min (isothermal). Then the temperature was programmed to 240°C at a rate of 3°C/min and kept constant at 240 °C for 10 min (isothermal). Finally, the temperature was programmed to 300°C at a rate of 4°C/min and kept constant at 300°C for 10 min (isothermal). The injector temperature was 280 °C. Helium carrier gas flow rate was 1.41 ml/min. All the mass spectra were recorded applying the following condition: (equipment current) filament emission current, 60 mA; ionization voltage, 70 eV; ion source, 220°C. Diluted samples (1% v/v) were injected with split mode (split ratio 1: 15) (Politz et al., 2013).

Results and Discussion

The ACo gene was transformed into *Zea mays* lines using chitosan nanoparticles. The transgenic plants expressed ACC enzyme, which initiates the production of fatty acids to produce biodiesel. ACo was isolated from *E. coli*, then obtained using PCR with their specific primers. After that, the PCR product of each gene separately was ligated into pMini-T vector. Then, the recombinant plasmids were transformed into competent 10-beta *E. coli* (Fig. 1). The colony PCR was performed to confirm ACo transformation into 10-beta *E. coli* for amplification. ACo was detected nearly at about 780 bp.

The transformation of ACo to increase the fatty acid content is familiar and applied in many types of research. Rudus´ et al. (2013) mentioned that ACC oxidase (ACO) is involved in the final step of ethylene production in plant tissues. Houben and Van de Poel (2019) highlighted the importance of ACO being a prime target for genetic engineering and precision breeding to control plant ethylene production levels. Fathy et al. (2021) transform ACo gene into some cyanobacterial species (*Synechocystis* sp.) to enhance the fatty acid contents in these species.

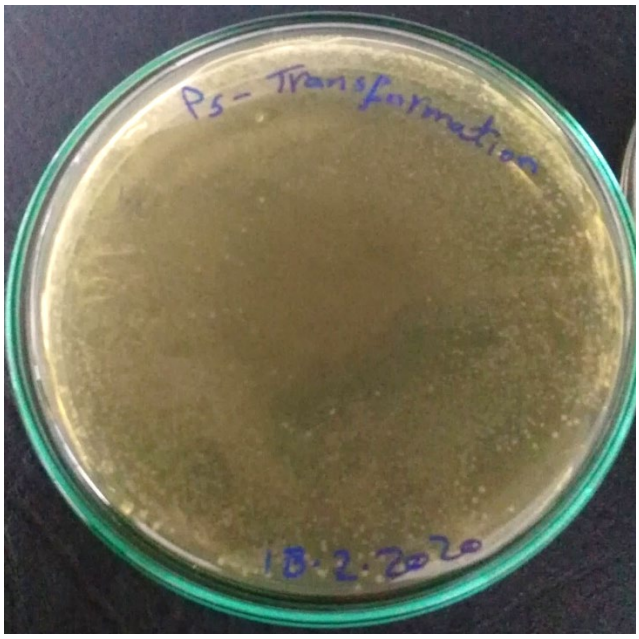

Figure 1. Transformation of *ACo* gene into 10-beta cells on LB supplemented with 100µg/L ampicillin.

Chitosan nanoparticle characterization

The characterization of chitosan nanoparticles included morphology by FT-IR, XRD, SEM measurements, zeta potential and zeta size. SEM images of the chitosan nanoparticles (CNPs) are shown in (Fig. 2) and illustrated that the nanoparticles had a solid and spherical with about 50 nm. Chitosan nanoparticles (CNPs) functional groups are studied using FTIR spectroscopy. The FTIR spectra of CNPs are shown in (Fig. 3) and indicating a peek at wavenumbers 3291 $cm^{-1,}$ indicating the stretching vibration of the $–NH_2$ and –OH groups. Other peaks at 1028 cm^{-1} showing P=O group, 1645 cm^{-1} for carbonyl group and 2921 for cm^{-1} (CH_3CH_2). The X-ray diffraction patterns taken for chitosan are presented in Fig. 4. Chitosan exhibited a characteristic crystalline peak at 2h= 20°, which was little shifted to a

higher diffraction angle. This indicated the better crystalline nature of the chitosan. The particle size distribution of the CNPs was measured by DLS and illustrate the Zeta potential (Fig. 5) and zeta size (Fig. 6).

Chitosan nanoparticle characterization is essential to estimate and define the variable characters of these nanoparticles. Mohanraj (2006) suggested that nanoparticles are solid particles with a size range of 10-1000 nm and the result of the current research is between the defined ranges. Saharan et al. 2015 and Choudhary et al. 2017 measured chitosan nanomaterials in water using DLS and found the ranges from 40 to 374 nm with an average size of ~250 nm. Rosyada et al. (2019) define chitosan nanoparticles to apply as an edible coating material. They used the following characterizations: particle size distribution and poly-dispersion index analysis and structural and spectral measurement using Fourier Transformed Infrared Spectroscopy (FT-IR).

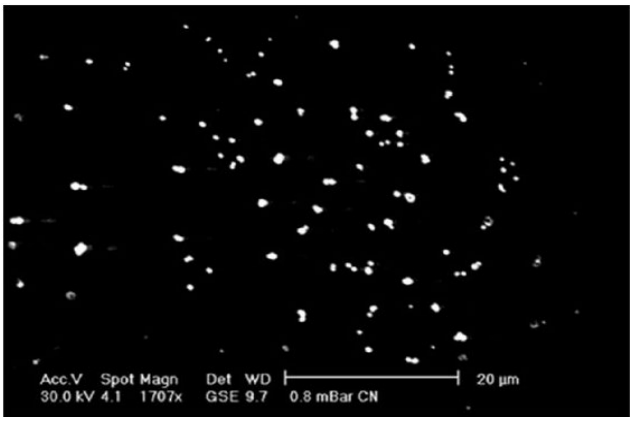

Figure 2. SEM of chitosan nanoparticle

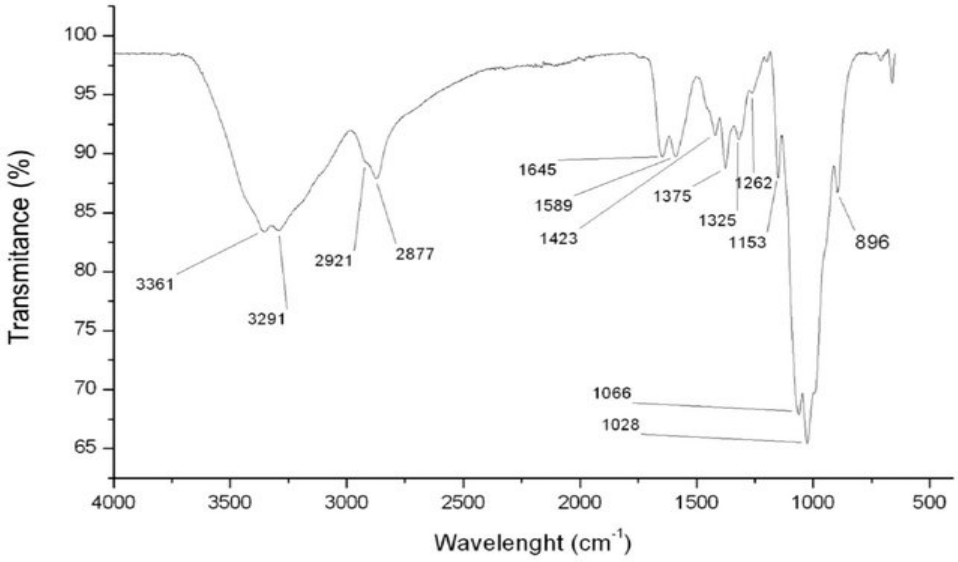

Figure 3. FT-IR of chitosan nanoparticle

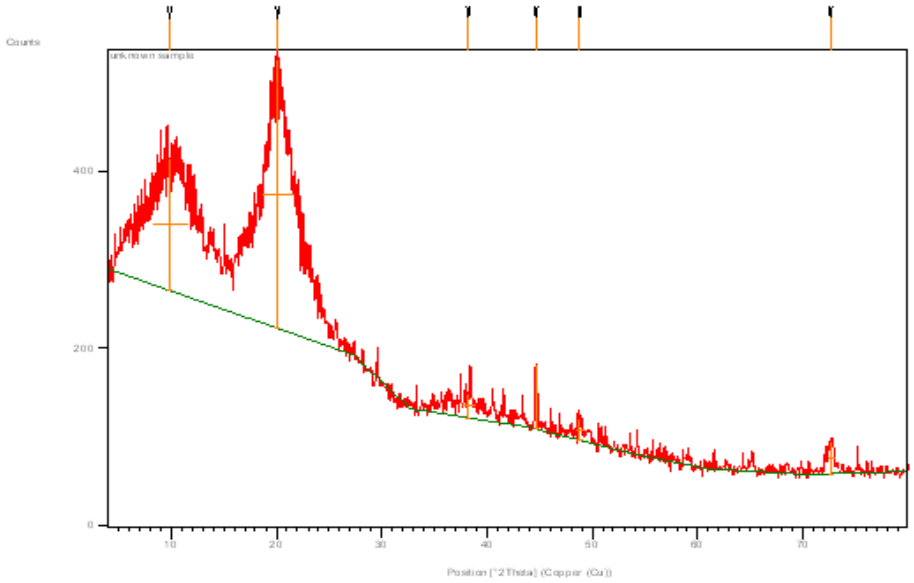

Figure 4. XRD of chitosan nanoparticle

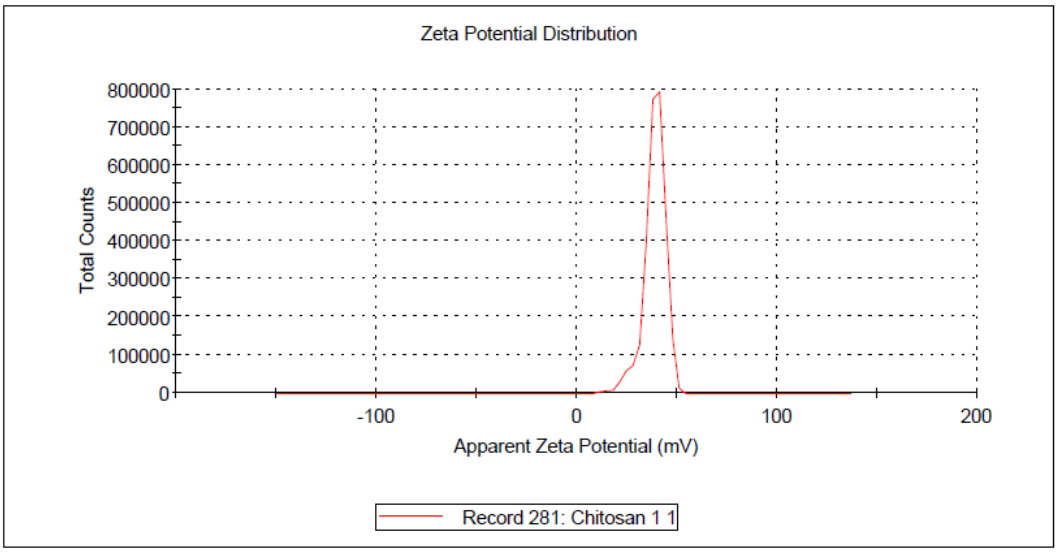

Figure 5. Zeta potential of chitosan nanoparticle

			Size (d.nm):	% Intensity:	St Dev (d.n...
Z-Average (d.nm):	173.3	Peak 1:	223.8	55.8	77.28
PdI:	0.268	Peak 2:	46.11	44.2	16.42
Intercept:	0.683	Peak 3:	0.000	0.0	0.000

Result quality : Refer to quality report

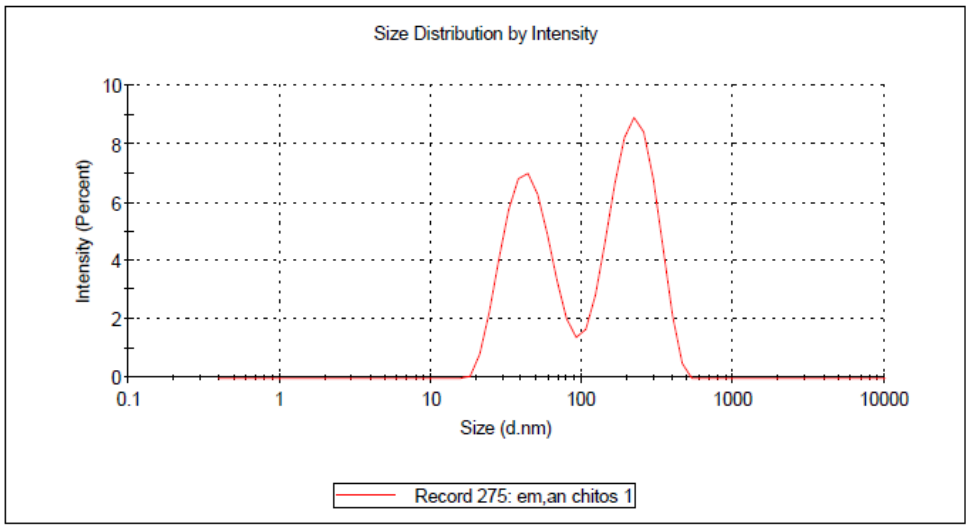

Figure 6. Zeta size of chitosan nanoparticle

CS/pDNA Transformation into Zea mays

After two weeks of seed germination, the CS/pDNA complex was inoculated in the maize seedlings. The regenerated transgenic and non-transgenic lines of *A. cepa* were left for more germination for another two weeks (Fig. 7). Confirmation of *ACo* gene transformation into *Z. mays* was applied using PCR for both transgenic and non-transgenic lines. The band was detected at 780 bp. The non-transgenic lines were applied to confirm that *ACo* gene was not found previously in this species (Fig. 8).

Chitosan nanoparticle is a modern technique used for transformation because it has many advantages: non-toxic, biocompatible, and biodegradable compared with the *Agrobacterium* technique. CS/pDNA complex was successfully prepared with defined conditions and used as an effective gene delivery system. Chitosan nanoparticles were used for the transformation of various genes in different sources like an animal and human cell lines, according to Kiang et al. (2004) and Hallaj-Nezhadietel et al. (2011). However, it is still limited to applied in gene transformation into plant tissues till Abdel-Razik et al. (2017), who used chitosan nanoparticles for gene transformation to potato lines. Also, this work agreed with Hussein (2020) who used

chitosan nanoparticle as a carrier of pMiniT carrying thionin gene through transformation into *Paulownia* sp. Finally, Fathy et al. (2021) transformed ACo gene into *Synechocystis sp.* using chitosan nanoparticles. Lv et al. (2020) mentioned that nanoparticles introduced new technology that improves gene transformation efficiency and overcomes difficulties in plant regeneration.

Total Lipids Profile for Transgenic and Non-Transgenic *Zea mays*

GC/MS analysis of fatty acids extracted from both Transgenic and Non-Transgenic *Zea mays* represented with total ion chromatogram in Figure (9a, b) and results compiled in Table (1) revealed the identification of 5 compounds in non-transgenic maize and 9 compounds in non-transgenic maize (Table 1). The four new fatty acids were 6-Methoxy-2-benzoxazolinone, Phytol, 1-Tridecanamine, N,N-dimethyl- and Stigmastan-6,22-dien, 3,5-dedihydro-. These new fatty acids in the transgenic maize could be due to the expression of ACC in maize. All these compounds represent more than 60% of the total peak area of the chromatogram.

Applying GC-MS for analyzing the variable fatty acids in maize plant, Maurya et al. (2016) examined lipid-extracted biomass residues of two microalgal species (*Chlorella variabilis* and *Lyngbya majuscule*) and evaluated for its nitrogen substitution potential for maize crop. Also, assess their effects on crop growth, yield, quality, and soil properties. Madany and Khalil (2017) evaluated the effect of fenugreek seed extract on the growth of faba bean (*Vicia faba*) and maize (*Zea mays*) seedlings. Shiva et al. (2018) tested the extract produced and used for analysis by mass spectrometry without a solvent evaporation step. The amount of lipid extracted, including phosphatidic acid, is comparable to widely used, more labor-intensive methods.

Figure 7. Transgenic and non-transgenic *Z. mays* lines

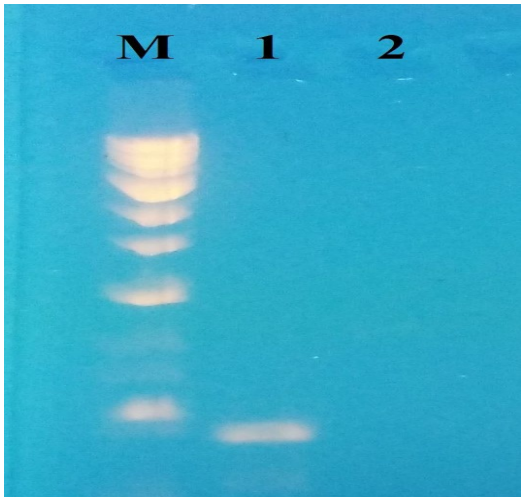

Figure 8. PCR confirmation for gene transformation into Zea lines. (1) Transgenic *Zea* lines with positive band for Acc gene, (2) Non-transgenic *Zea* lines with negative Acc gene band.

Table 1. Chemical composition of fatty acids of non-transgenic and transgenic *Zea mays*

No.	Compounds	Area %	
		Non-Transgenic maize	Transgenic maize
1	2-Tridecene, (Z)-	15.45	15.12
2	1-Octadecyne	19.60	20.1
3	1-Pentadecanamine, N,N-dimethyl-	22.54	22.91
4	Stigmasterol, 22,23-dihydro-	20.99	22.80
5	Hexadecanal	14.75	16.21
6	6-Methoxy-2-benzoxazolinone	-	36.37
7	Phytol	-	8.11
8	1-Tridecanamine, N,N-dimethyl-	-	4.65
9	Stigmastan-6,22-dien, 3,5-dedihydro-	-	9.20

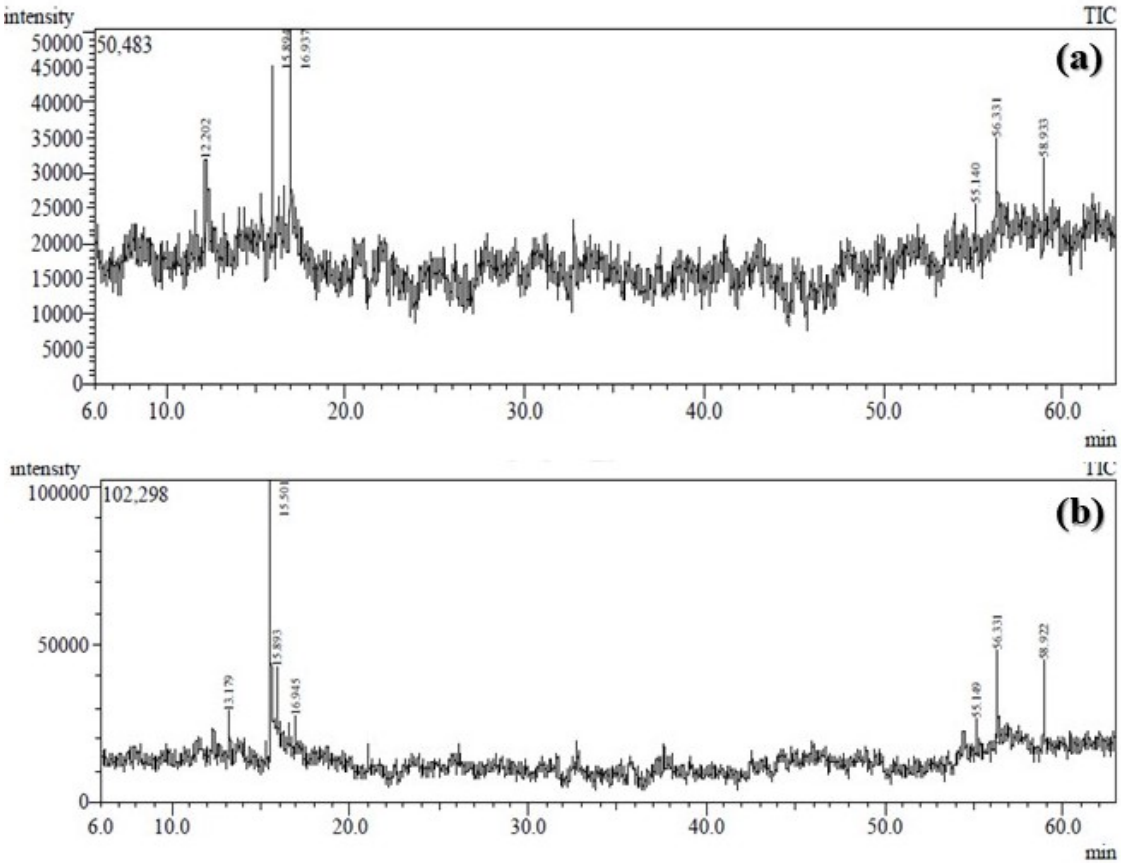

Figure 9. GC-MS Chromatogram of fatty acids of (a) non-transgenic *Zea mays*, and (b) transgenic *Zea mays* expressing *ACo gene*.

Conclusion

Zea mays (corn) is an essential crop for animal and human feeding and it is a good source of essential fatty acids due to its excellent nutritional properties. Acetyl-CoA gene is translated into Acetyl-CoA carboxylase, which is the first step in fatty acid production. Fatty acid production is an essential and primary step for biofuel production. In this study, we transform the Acetyl-CoA gene (Aco) loaded on chitosan nanoparticles to enhance the fatty acid production in the maize plant. Chitosan nanoparticles provide a direct, efficient gene injection method in plant tissues due to the high affinity of chitosan to the plasmid carrying the target gene. The chitosan nanoparticles were characterized using SEM, XRD, FT-IR and Zeta potential and size. Besides, this gene increases the production of maize fatty acids, but also help in the production of new fatty acids after GC-MS analysis. To confirm the transformation of ACo gene into transgenic maize lines, conventional PCE was applied compared to non-transgenic lines.

Acknowledgment

The authors would like to thank Dr. Ahmed Hassan (Botany and Microbiology Department) and Dr. Salim Mohammed (Chemistry Department), Faculty of Science, Helwan University; Mr. Wael Fathy (Assistant lecturer, Faculty of Science, Beni Suef University) for their help during practical work and data explanation. Also, we would like to thank Prof. Dr. Ashraf Bakry (Professor of Genetics, Faculty of Agriculture, Ain Shams University) for supporting the chitosan nanoparticles.

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