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.

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Introduction

Zea mays (L.), also known as corn, is a monocot cereal grain with a leafy stalk that produces ovoliferous 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 (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%

Maize has a high nutritional value. Maize grains have a high nutritional value. Maize grains have a high nutritional value 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 ddH2O. The primers were designed using snap gene® (2.3.3) software; ACC-F: 5′-GACCTCTGTCCCTCCCTGAC-3′, ACC-R: 5′-CCGCCGTTTGCAAAACAAAT-3′. PCR program was repeated 40 cycles as follows: 30 sec at 95°C, 30 sec at 50°C, and 1 min at 72°C. 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 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% (w/v) agarose 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 Å) (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 55°C for 15 min and then added equally to each other with stirring on a vortex for 1 min.

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 peak at wavenumbers 3291 cm⁻¹, indicating the stretching vibration of the –NH₂ and –OH groups. Other peaks at 1028 cm⁻¹ showing P=O group, 1645 cm⁻¹ for carbonyl group and 2921 for cm⁻¹ (CH₃CH₂). 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
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 Stigmaster-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](image)

![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.](image)

| 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   | Stigmasteran-6,22-dien, 3,5-dedihydro-       | -               | 9.20            |

**Table 1. Chemical composition of fatty acids of non-transgenic and transgenic *Zea mays***
Transgenic Maize Enhancing Fatty Acid Production

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.

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References

1. Abdel-Razik AB, Hammad IA, Tawfik E. Transformation of Thionin Genes Using Chitosan Nanoparticle into Potato Plant to Be Resistant to Fungal Infection. IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB). 2017; 3(3): 1–13.
2. Choudhary RC, Kumaraswamy RV, Kumari S, Sharma SS, Pal A, Raliya R, Biswas P, Saharan V. Synthesis, characterization, and application of chitosan nanomaterials loaded with zinc and copper for plant growth and protection. In: Prasad et al (eds) Nanotechnology. Springer Nature Singapore Pte Ltd, Singapore. 2017.
3. Edwards K, Johnstone C, Thompson C A Simple and Rapid Method for the Preparation of Plant Genomic DNA for PCR Analysis. Nucleic Acid Res. 1991; 19:1349.
4. Fathy W, Essawy E, Tawfik E, Khedr M, Abdelhameed MS, Hammouda O, Elsayed K. Recombinant
overexpression of the *Escherichia coli* acetyl-CoA carboxylase gene in *Synechocystis* sp. boosts lipid production. *J Basic Microbiol.* 2021. DOI: 10.1002/jobm.202000656

5. Gheṭe AB, Duda MM, Várban DI, Várban R, Moldovan C, Muntean S. Maize (*Zea mays*), A Prospective Medicinal Plant in Romania. *Hop and Medicinal Plants.* 2018; 26(1-2): 44-51.

6. Hallaj-Nezhadietal S, Valizadeha H, Dastmalchiesa S, Baradaranc B, Jalalia MB, Dobakhtid F, Lotipouref. Preparation of Chitosan-Plasmid DNA Nanoparticles Encoding interleukin-12 and their Expression in CT-26 Colon Carcinoma Cells. *J Pharm Pharmaceut Sci* 2011; 14(2): 181-195.

7. Houben M, Van de Poel B. 1-Aminocyclopropane-1-Carboxylic Acid Oxidase (ACO): The Enzyme That Makes the Plant Hormone Ethylene. *Front Plant Sci.* 2019.10:695. doi: 10.3389/fpls.2019.00695

8. Hussien ET. Production of transgenic Paulownia tomentosa (Thunb.) steud. using chitosan nanoparticles. *Biotechnol Biochem*. 2020; 9(2): 55-62.

9. Kavipriya C, Yuvaraja A, Senthil K, Menaka C. Genetic Transformation Methods for Crop Improvement: A Brief Review. *Agric Rev.* 2019; 40(4): 281-288.

10. Kiang T, Wen J, Lim HW, Leong KW. The effect of the degree of chitosan deacetylation on the efficiency of gene transfection. *Biomaterials Elsevier Ltd.* 2004; 25: 5923-5931.

11. Konishi T, Shinhoara K, Yamada K, Sasaki Y. Acetyl-CoA carboxylase in higher plants; most plants other than Gramineae have both the prokaryotic and the eukaryotic forms of this enzyme. *Plant Cell Physiol.* 1996; 37: 117–122.

12. Li G, Wang J, Feng X, Liu Z, Jiang C, Yang J. Preparation and Testing of Quaternized Chitosan Nanoparticles as Gene Delivery Vehicles. *Appl Biochem Biotechnol.* 2015; 175: 3244–3257.

13. Lv Z, Jiang R, Chen J, Chen W. Nanoparticle-mediated gene transformation strategies for plant genetic engineering. *Plant J.* 2020; 104: 880–891

14. Madany MMY, Khalil RR Fenugreek Seed Extract Enhanced the Growth of *Vicia faba* and *Zea mays* Seedlings. *Egypt J Bot.* 2017; 57(2): 363- 377.

15. Mansouri S, Cuie Y, Wimmik F, Shi Q, Lavigne P, Benderdour M, Beaumont E. Characterization of folate-chitosan-DNA nanoparticles for plant genetic engineering. *Plant J.* 2020; 105). New Delhi: Krishi Sanskriti Publications.

16. Maurya R, Chokshi K, Ghosh T, Trivedi K, Pancha I, Kubavat D, Misra S and Ghosh A. Lipid Extracted Micropalgal Biomass Residue as a Fertilizer Substitute for *Zea mays*. *Front Plant Sci.* 2016; 6:1266. doi: 10.3389/fpls.2015.01266

17. Mohanraj VJ, Chen Y. Nanoparticles-a review *Trop J Pharm Res.* 2006; 5: 561-573.

18. Murdia LK, Wadhvani R, Wadhawan N, Bajpai P, Shekhawat S. Maize Utilization in India: An Overview. *American Journal of Food and Nutrition (AJFSN).* 2016; 4(6): 169-176

19. Periayah MH, Halim AS, Saad AZ. Chitosan: A Promising Marine Polysaccharide for Biomedical Research. *Phcog Rev.* 2016; 10:39-42.

20. Politz M, Lennen R, Pfleger B. Quantification of Bacterial Fatty Acids by Extraction and Methylation. *Bio-protocol.* 2013; 3(21): e950. DOI: 10.21769/ BioProtoc.950.

21. Raftery R, O’Brien FJ, Cryan SA. Chitosan for Gene Delivery and Orthopedic Tissue Engineering Applications. *Molecules* 2013; 18: 5611-5647.

22. Rosyada A, Sunarharum WB, Waziriho E. Characterization of chitosan nanoparticles as an edible coating material. International Conference on Green Agro-industry and Bioeconomy. *IOP Conf. Series: Earth and Environmental Science.* 2019; 230, 012043. doi:10.1088/1755-1315/230/1/012043.

23. Rudus’ I, Sasiak M, Kepczynski J. Regulation of ethylene biosynthesis at the level of 1-aminocyclopropane-1-carboxylate oxidase (ACO) gene. *Acta Physiol Plant.* 2013; 35:295-307.

24. Saharan V, Sharma G, Yadav M, Choudhary MK, Sharma SS, Pal A, Raliya R, Biswas P. Synthesis and in vitro antifungal efficacy of Cu-chitosan nanoparticles against pathogenic fungi of tomato. *Int J Biol Macromol.* 2015; 75:346–353.

25. Sasaki Y, Nagano Y. Plant Acetyl-CoA Carboxylase: Structure, Biosynthesis, Regulation, and Gene Manipulation for Plant Breeding. *BioSci Biotechnol Biochem.* 2004; 68 (6): 1175-1184.

26. Seda Tiği R, Karakeçili A, Gümüşderelioglu M. In vitro characterization of chitosan scaffolds: Influence of composition and deacetylation degree. *J Mater Sci Mater Med.* 2007; 18:1665-74.

27. Shah TR, Prasad K, Kumar P. Studies on physicochemical and functional characteristics of asparagus bean flour and maize flour. In G. C. Mishra (Ed.), Conceptual frame work & innovations in agroecology and food sciences. 2015. (Ist ed., pp. 103–105). New Delhi: Krishi Sanskriti Publications.

28. Shigemasa Y, Matsuura H, Sashii H, Saimoto H. Evaluation of different absorbance ratios from infrared spectroscopy for analyzing the degree of deacetylation in chitin. *Int J Biol Macromol.* 1996; 18:237-42.

29. Shiva S, Enminful R, Roth MR, Tamura P, Jagadish K, Welti R. An efficient modified method for plant leaf lipid extraction results in improved recovery of phosphatidic acid. *Plant Methods.* 2018; 14: 14.

30. Siafu SI. Silicone Doped Chitosan-Acrylamide Coencapsulated Urea Fertilizer: An Approach to Controlled Release Fertilizers. *J Nanotechnol.* 2017; Article ID 8490730, 7 pages. https://doi.org/10.1155/2017/8490730

31. Zhang J, Xia W, Liu P, Cheng Q, Tahirou T, Gu W, et al. Chitosan modification and pharmaceutical/bio-medical applications. *Mar Drugs.* 2010; 8: 1962-1987.