Genetics & Applications

Institute for Genetic Engineering and Biotechnology University of Sarajevo

Research Article Open access

CRISPR/Cas9-mediated targeted mutagenesis of FAD2-1 gene for oleic acids composition in sunflower

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DOI: 10.31383/ga.vol6iss2ga09

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Received
November, 2022
Accepted
December, 2022
Published
December, 2022

Keywords

Sunflower, CRISPR/Cas9, Genome editing, Oleic acid, FAD2-1 gene, Agrobacterium-mediated transformation

Abstract

Sunflower (*Helianthus annuus* L.) is the most widespread plant used for production of oil among all oilseeds cultivated in Turkey in terms of agricultural area and production. Sunflower with high oleic acid content is always desirable because of benefits on health and industrial use. The enzyme *FAD2-1* catalyzes the conversion of oleic acid to linoleic acid in sunflower. The fatty acid composition can be enhanced by gene editing of *FAD2-1* gene by CRISPR/Cas9 technique which has been applied recently as a new breeding technique to improve productivity and enhance sustainability in agriculture. Hence, CRISPR/Cas9 genome editing systems were utilized to knockout the *FAD2-1* gene in order to increase production of oleic acid content. For this purpose, two low oleic sunflower genotypes were transformed with two sgRNA that target *FAD2-1* gene. sgRNA expression cassettes were assembled into the binary vector by Golden Gate assembly in a single reaction which is followed by *A. tumefaciens*-mediated transformation and *in vitro* germination. Putatively transformed shoots were selected with *A. tumefaciens*-mediated the optimized kanamycin concentration (100 mg/L) in the medium. The challenges of transformation of sunflower were summarized and possible solutions were proposed. This study indicated that sunflower still can be modified by CRISPR/Cas9 genome editing system to develop high oleic sunflower.

Introduction

Sunflower (*Helianthus annuus* L.) is one of the world's most significant oilseed crops with various usage areas (Hu et al., 2010). Sunflowers are categorized in terms of fatty acid composition into high oleic (85%), mid oleic (60-65%), and linoleic (low-oleic).

The demand for both production and consumption of high oleic sunflowers has increased in the world, as high oleic sunflower genotypes have various advantages in terms of their valuable oil content for industrial use and human health (Kaya et al., 2007).

The oil composition of sunflower can be altered by the genetic modifications made on the fatty acid desaturases 2 (*FAD2*) gene that facilitates the biological conversion of oleic acid to linoleic acid. Oleic acid composition was elevated up to 75% in Pervenets variety of sunflower with the application of dimethylsulphoxide (DMSO) which is a chemical mutagen (Soldatov et al., 1976).

Many inbred lines that are derived from the mutant Pervenets had oleic acid composition up to 90% (Fernandez-Martinez et al., 1993; Miller et al., 1987; Zambelli et al., 2015). Besides, Vick & Miller (1996) reported the development of the high and mid oleic sunflower mutants by treatment with Ethyl-Methane Sulphonate (EMS). Similarly, EMS treatment was also performed by Leon et al. (2013b) to develop high oleic acid mutants. The treatment induced point mutations causing amino acid substitutions and premature stop codon (Leon et al., 2013b).

On the other hand, the duplication of the *FAD2-1* gene leads to the transcription silencing of the gene and consequently, accumulation of oleic acid (Lacombe et al., 2009; Martinez-Rivas et al., 2001). In addition, Schuppert et al. (2006) also reported that high oleic mutant sunflowers were developed by duplication of the *FAD2-1* gene and oleoylphophatidyl choline desaturase induction in sunflower genotypes.

Differently, Leon et al. (2013a) developed two high oleic sunflower genotypes by X-rays treatment of the seeds. The treatment resulted in gene insertions in different regions of the genomes that caused the generation of the premature stop codon and thereby accumulation of oleic acid.

Besides the application of chemical mutagens and duplication studies, the recently developed CRISPR/Cas9 technique provides a useful, quick, effective and, more importantly, precise genome editing toolbox and has been applied as a new breeding technique to improve productivity and enhance sustainability in agriculture (Chen et al., 2019).

Thus, CRISPR/Cas9-mediated targeted mutagenesis of the *FAD2* gene for the enhancement of oleic acid production has been studied in a variety of plants. For instance, Jiang et al. (2017) edited *FAD2* gene in *Camelina sativa* L. for the improvement of fatty acid composition. Gene silencing of the *FAD2* gene resulted in an increase of the oleic acid content from 16% to 50%.

Abe et al. (2018) also improved fatty acid composition by knocking out *FAD2* gene in rice while Tian et al. (2020) knocked out *FAD2-2* gene in tobacco (*Nicotiana tabacum* L.).

Yuan et al. (2019) also accomplished targeted mutagenesis of *FAD2A* and *FAD2B* genes in peanut seeds. Moreover, Okuzaki et al. (2018) also edited *FAD2* gene in *Brassica napus* L. and accomplished the fatty acid composition to be significantly increased.

Al Amin et al. (2019) developed high oleic soybean by silencing of *FAD2-2* gene with 21% mutation efficiency.

Since the previous studies indicated that knocking out *FAD2* gene by CRISPR-Cas9 resulted in elevation of the fatty acid content in significant number of plants, the aim of the study concerned the application of this gene editing technique to knock out *FAD2-1* gene in sunflower (*H. annuus* L.).

Material and methods

Plant material

Sunflower (*H. annuus* L.) seeds with low oleic fatty acid content were used as the starting material in the present study. The seeds of *H. annuus* L. lines (2453-A and Deray) were provided by the Republic of Türkiye Ministry of Agriculture and Forestry, Trakya Agricultural Research Institute, Edirne, Turkey. The seeds were planted in soil and grown in a growth chamber at Marmara University, Faculty of Arts and Sciences, Biology Department, Plant Biotechnology Laboratory. The growth conditions were optimum at 25-26°C, 60% humidity, a light intensity of about 6000 lux, and a photoperiod with 16 hours of light and 8 hours of darkness.

Verification of FAD2-1 Gene in Sunflower

The genomic DNA was extracted from the leaf samples harvested from two weeks old Deray and 2453A sunflower plants by Doyle and Doyle (1987) CTAB method. Spectrophotometric analysis and gel electrophoresis were performed to determine the concentration and purity of the DNA extract. Target-specific primers for the amplification of the *FAD2-1* gene were designed using Primer3Plus Software (Rozen and Skaletsky, 2000) and synthesized by Sentegen Biotech (Turkey). The sequence of interest was amplified using a primer set (5'-CGCTAACCCGTTCGTTCTCC-3', 5'-ATCATAATGCGGCAAGCCAG-3') by following PCR conditions 98°C for 30s followed by 30 cycles of 98°C for 10 s, 56°C for 15 s, 72°C for 30 s, and final extension at 72°C for 5 min for *FAD2-1* gene amplification. PCR was carried out in a total of 50 µl consist of 0.5 µl Phusion® High Fidelity Polymerase (2U/µl), 10 µl 5X Phusion® HF Buffer (0.02 U/µl), 2.5 µl Primer Forward (10 µM), 2.5 µl Primer Reverse (10 µM), 4 µl dNTPs (10 mM), 2 µl Template DNA (100 ng/µl), 28.5 µl PCR grade water. The amplified products were sequenced by Sanger method with service procurement.

Gene Editing of FAD2-1 gene

Single guide RNA (sgRNA) design

sgRNA targeting Delta-12 oleate desaturase of *H. annuus* L. (*FAD2-1*) (Chapman, 2012) was designed using guide RNA prediction algorithms of CRISPOR-Tefor Software (http://www.crispor.tefor.net) and gRNAs with minimum off-target mismatches counts were chosen. NEBcutter V2.0 program was used to map restriction enzymes with their cleavage codes on the sequence.
Then, The mFold Web Server RNA Folding Version 2.3 Energies (http://unafold.rna.albany.edu/?q=mfold/RNA-Folding-Form2.3) (Zuker, 2003) was used to exclude the candidate sequences that are likely to form hairpins or stems longer than 6 base pairs with the sgRNA.

Vector construction and cloning to *E. coli*

The assembly of the expression cassettes into pYLCRISPR/Cas9 binary plasmid was carried out according to Golden Gate Assembly method (Engler et al., 2009) and pHDE-35SCas9-mCherry plasmid by Gibson Assembly method (Gibson et al., 2008). The adaptor sgRNA primers were designed by adding 5'-GTCA-3' (for U3 promoter) and 5'-ATTG-3' (for U6 promoter) onto the 5' end of the sgRNA sequences that were designed and obtained. Digestion/ligation reaction was performed for each sgRNA expression cassette. pYLsgRNA-AtU3d/LacZ plasmid (Addgene with #66201 catalog number) and pYLsgRNA-AtU6-1 plasmid (Addgene with #66202 catalog number) were used in the reaction. The initial concentrations of pYLsgRNA U6 and pYLsgRNA U3 cassette plasmids were 226.12 ng/µl and 155 ng/µl respectively. The cassette plasmids were diluted to 20 ng/µl (1:11 and 1:9 dilutions). The reaction mixes for 10 µl reactions

were prepared in microcentrifuge tubes (0.2 mL) for both U3 and U6 primer mixes. After the reaction was done, the ligation products were 1:10 diluted and used in the first PCR with U-F (5' CTCCGTTTTACCTGTGGAATCG 3') and gR-R (5' CGGAGGAAAATTCCATCCAC 3') primers. PCR products were directly used as the template DNA for the second PCR (overlapping PCR) to make the expression cassettes at the end. The arrangements of Pps/Pgs primer pairs for the two cassettes were as follows: Pps-R/Pgs-2, Pps-2/Pgs-L for the use of pYLCRISPR/Cas9P35S-N and Pps-PmeI-F/Pgs-2, Pps-2/Pgs-SpeI-R for the use of pHDE-35SCas9-mCherry vector. The sequences of the primers used were given in the Table 1. Each PCR product (10 µl) were run on 2% agarose gel electrophoresis to verify the size. The expected size of sgRNA cassettes was 495 bp (360bp + 135 bp). DNA Clean & Concentrator™ - 5 Kit (Zymo Research, catalog no: D4013) was used for the purification of each overlapping PCR product. U3-sgRNA and U6-sgRNA expression cassettes were obtained in the second (Overlapping) PCR and used as DNA templates in Gibson Assembly protocol. First, the second PCR products were cut at *BsaI* recognition site and combined by PCR reaction. The PCR product was purified with DNA Clean & Concentrator™ - 5 Kit and DNA concentration was quantified using NanoDrop spectrophotometer. Another PCR reaction was performed to generate sticky and blunt ends for SpeI and PmeI restriction enzymes on the previous U3/U6-sgRNA PCR product. The PCR product was purified and quantified. NEBioCalculator (https://nebiocalculator) was used to calculate the required mass of insert DNA. According to the result given by NEBioCalculator, 30 ng DNA insert was used for 100 ng vector. The U3/U6 sgRNA fragment was cloned between the *PmeI* and *SpeI* sites in pHDE-35SCas9-mCherry CRISPR/Cas9 vector (Gao et al., 2016) by Gibson Assembly. Similarly, U3-sgRNA and U6-sgRNA expression cassettes were prepared in Second (Overlapping) PCR and used as DNA templates in Golden Gate Assembly protocol. The U3/U6 sgRNA fragment was cloned at *BsaI* sites into pYLCRISPR/Cas9P35S-N vector (Ma et al., 2015) by Golden Gate Assembly. The PCR reaction for the ligation of DNA insert into the vector was performed. The digestion/ligation mix was prepared. The competent *E. coli* cells were prepared according to Chung et al. (1989). 100 µl competent cells and 4 µl ligation product were mixed in a microcentrifuge tube and mixed gently by flicking the bottom of the tube with finger. The mixture was kept on ice for 5 minutes and then, it was incubated at 42°C in water bath for 60 seconds and transferred on ice for 5 minutes. Then, the mixture was added into 1 mL LB (Luria-

Table 1. Primers used in the second PCR (Overlapping PCR)

Primer designation	**Sequence (5'→3')**
Pgs-2	AGCGTGGGTCTCGTCAGGGTCCATCCACTCCAAGCTC
Pps-2	TTCAGAGGTCTCTCTGACACTGGAATCGGCAGCAAAGG
Pps-R	TTCAGAGGTCTCTACCGACTAGTATGGAATCGGCAGCAAAGG
Pgs-L	AGCGTGGGTCTCGCTCGACGCGTATCCATCCACTCCAAGCTC
Pgs-*SpeI*-R	GTACTAGTATCCATCCACTCCAAGCTC
Pgs-*PmeI*-F	AGTTTAAACATGGAATCGGCAGCAAAGG

Bertani) liquid medium and incubated for 1 hour at 37°C while shaking at 200 rpm. After incubation, the mixture was centrifuged at 800 x g for 5 minutes. The supernatant was poured out, and the pellet was re-suspended in the remaining supernatant. The *E. coli* cells were spread on LB agar supplemented with 50 mg/L kanamycin and 40 µl of both IPTG (100 µM) and X-gal (20 mg/mL). The agar plates were incubated at 37°C for 24 hours. The presence of insert DNA in the plasmid construct was determined by colony PCR (Woodman, 2008). After the verification of the presence of DNA insert by gel electrophoresis, a sizeable colony was picked with a 100 µl pipette tip and added into 5 mL LB medium supplemented with 5 µl kanamycin (50 mg/mL). The culture was covered loosely with a cap that is not air-tight and incubated at 37°C for 12-16 hours in a shaking incubator. Zyppy Plasmid Miniprep Kit (Zymo Research, catalog no: D4019) was utilized in the plasmid isolation. The plasmid DNA was quantified using NanoDrop Lite Spectrophotometer. Later, the entire plasmid DNA was sequenced.

A. tumefaciens transformation

A. tumefaciens tumefaciens LBA4404 and EHA105 strains were kindly provided by Dr. Allahbakhsh (Niğde Ömer Halis Demir University, Turkey) and Dr. Elisabeth Chevreau (INRAE – French National Institute for Agriculture, Food and Environment, France).

Freeze-thaw transformation protocol was applied. In summary, 100 µl competent cells A. tumefaciens were thawed in a 1.5 mL microcentrifuge tube on ice. Plasmid DNA (~1.0 µg) was added into the competent cells and mixed by tapping the tube gently. The plasmid DNA-competent cells mixture was incubated in liquid nitrogen for 5 minutes and then, incubated in 37°C water bath for 5 minutes. The mixture was tapped gently and kept on ice for 2 minutes. Additional 1 mL liquid LB was added to the tube and incubated for 2 hours at 28°C with shaking (200 rpm). The mixture was centrifuged at 3000 rpm for 5 minutes. Approximately 1 mL of supernatant was removed and the bacterial pellet was re-suspended in the remaining supernatant. The *A. tumefaciens* cells were spread on LB agar plates supplemented with antibiotics. Gentamycin (30 µg/mL) and kanamycin (100 µg/mL) were used for EHA105 strain. Streptomycin (200 µg/mL) and kanamycin (100 µg/mL) were used for LBA4404 strain. The agar plates were incubated at 28°C for 2 days. The glycerol stock of confirmed *A. tumefaciens* transformants was prepared by mixing the fresh bacterial suspension with 30% glycerol in 1:1 ratio. The bacterial glycerol stock was stored at -80°C.

Sunflower transformation

The frozen aliquot of *A. tumefaciens* which was stored at -80°C, was added into 50 mL of LB broth medium including 50 µg/mL kanamycin and swirled. The culture was covered loosely with sterile aluminum foil. The bacterial culture was incubated overnight at 28°C while shaking (230 rpm) in the shaking incubator. After overnight incubation, the bacterial culture was transferred into 50 mL Falcon centrifuge tube and centrifuged at 2800 x g at 4°C for 20 minutes. The supernatant was discarded without disturbing the bacterial pellet and the bacterial pellet was dissolved in 50 mL of liquid half-MS medium. The density of the bacterial culture was measured at OD600 in spectrophotometer (Nanophotometer® 330 by Implen). The bacterial culture was diluted to 0.6 OD600 with liquid half-MS medium and 150 µM acetosyringone (Sujatha et al., 2012). The sunflower seeds were sterilized according to Dagustu et al. (2010). The sterilized seeds were split into two halves, longitudinally along the embryo axis. The half-seeds were added into *A.*

tumefaciens culture with 0.6 OD600 and subjected to vacuum infiltration for 10 minutes at 60 kPa. The half-seeds were placed on filter paper for drying. Thereafter, the half-seeds were co-cultured with *A. tumefaciens* cells (Sujatha et al., 2012).

Following the sunflower transformation, the half-cut seeds which were inoculated with *A. tumefaciens* cells, were removed from the bacterial culture and blotted dry on a filter paper and co-cultivated in co-cultivation medium [MS (Murashige and Skoog, 1962) containing 0.5 mg/L BAP, 0.2 mg/L NAA, and 20 mg/L acetosyringone] in dark at 26 ± 2°C for 2 days. Following co-cultivation, the half-cut seeds were washed with 25 mL ½ MS broth medium including 250 mg/L cefotaxime, three times for 5 minutes each for elimination of *A. tumefaciens* cells. Then, the half-seeds were transferred to Selection 1 medium (SM1, MS medium containing 0.2 mg/L BAP, 0.01 mg/L NAA, 0.82 mg/L AgNO3, 250 mg/L cefotaxime, and 1 mg/L kanamycin) for shoot induction and the adaxial surface of the seeds was placed in contact with the medium surface. After 2 weeks of cultivation, the shoots were transferred to Selection 2 medium (SM2, MS medium containing 0.2 mg/L BAP, 0.82 mg/L AgNO3, 250 mg/L cefotaxime, and 10 mg/L kanamycin) for two weeks which is followed by their transfer to Selection 3 medium (SM3, MS medium containing 0.1 mg/L BAP, 0.82 mg/L AgNO3, 250 mg/L cefotaxime, and 50 mg/L kanamycin) for shoot elongation. The elongated shoots were transferred to rooting medium (RM, MS medium containing 1.0 mg/L NAA and 250 mg/L cefotaxime) and cultured for 20-25 days. The rooted shoots were transferred to soil and placed in the greenhouse to be acclimated. The plantlets were maintained under high humidity for one week (Sujatha et al., 2012).

Analysis of putative transformed shoots by PCR

For molecular analysis of putative transformed shoots, the shoots were harvested. Doyle & Doyle (1987) protocol was used for the genomic DNA extraction from the leaves. The target gene *FAD2-1*, was amplified by PCR using target specific primers and then sequenced. In addition, the presence of kanamycin resistance gene was also verified by PCR using KANF (5' CTATTCGGCTATGACTGGGC 3') and KANR (5' AGGCATCGCCATGTGTCACG 3') primers. The PCR products were run on 2% agarose gel.

Moreover, in order to confirm T-DNA integration, another PCR reaction was performed with using primers from both inside:

Pps F1

5'TTCAGAGGTCTCTCTGACACTGGAATCGG CAGCAAAGG 3' and

PgsR2

5'AGCGTGGGTCTCGTCAGGGTCCATCCACT CCAAGCTC 3' and outside:

PmeI

5' TGGACGAACGGATAAACCTT 3' of the T-DNA region

Data collection and analyses of A. tumefaciens-mediated sunflower transformation efficiency

Two technical replicates were used in in vitro germination studies. The percentage rates of survival of half-seeds co-cultured with *A. tumefaciens* cells in co-cultivation medium were calculated. In addition, the results of shoot and root formations in SM1, SM2, SM3, and RM were recorded. Co-cultivation was repeated twice for each cultivar (total 4 transformation groups) with 150 half cut seeds in each. In total, 600 half-seeds were used for agroinfection.

Results and Discussion

FAD2-1 gene amplication and sequencing

The approximate length of PCR fragments obtained by amplification of *FAD2-1* gene was between 700-

750 bp (Figure 1). The standard curve was made in Microsoft Excel to determine the length of DNA fragments according to the 1kb and 100 bp DNA Ladder. The determined size of DNA fragment was 718 bp. Then, DNA fragments were sequenced and no SNPs were found. Thus, it was confirmed that FAD2-1 gene sequences were the same in both genotypes and correspond to the sequences available in NCBI database. The sequence of *FAD2-1* gene that was amplified from Deray and 2453-A genotype was given in Figure 2.

Gene Editing of FAD2-1 Gene

A variety of factors including utilization of two different *A. tumefaciens* strains (EHA105 and LBA4404), two different low oleic genotypes of sunflower (2453-A and Deray), and two different assembly techniques (Golden Gate and Gibson assembly) was assessed to increase transformation efficiency in this study.

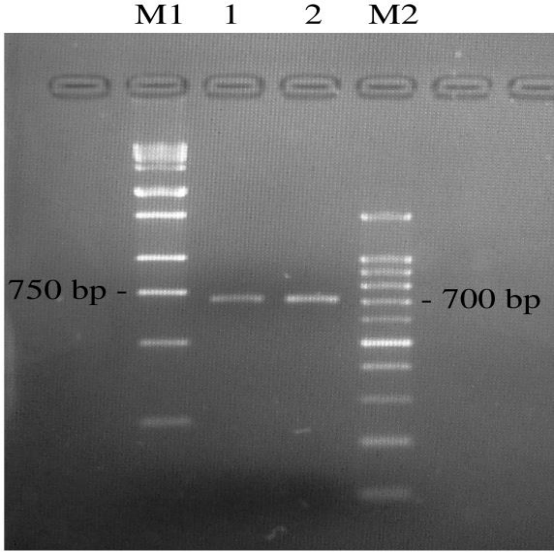

Figure 1. Agarose gel electrophoresis of FAD2-1 gene amplified fragment (1) Deray (2) 2453-A (M1) Sizer™ – 1 kb DNA Ladder (Intron) (M2) Sizer™ – 100 bp DNA Ladder (Intron).

GCCTCCATAACCGCTGTCCTCTACCACATTGCCACCACCTACTTCCACCACCTCCCCACCCCTTTGTCATCCATC
GCATGGGCCTCTTACTGGGTAGTCCAAGGCTGCGTCCTCACCGGAGTCTGGGTCATCGCCCACGAATGTGGTCAC
CATGCGTTTAGTGATTATCAATGGGTCGACGACACTGTGGGCTTTGTTCTCCACTCGTCTTTACTCGTCCCTTAC
TTTTCGTGGAAATATAGTCACCACCGCCACCATTCCAACACTGGATCACTCGAGCGGGACGAGGTTTTCGTCCCC
AAATCCCGATCGAAAGTCCCGTGGTACTCGAAATACTTTAACAACACAGTGGGCCGCATTGTCAGTATGTTCGTC
ACTCTCACTCTCGGCTGGCCCTTGTACTTAGCTTTCAATGTGTCGGGCCGACCCTATGACCGTTTCGCCTGCCAC
TACGTCCCAACCAGCCCTATGTACAATGAACGTAAACGTTACCAGATAGTCATGTCCGACATCGGGATTGTTATC
ACATCGTTCATCCTTTATCGTGTTGCTATGGCAAAAGGGTTGGTTTGGGTGATTTGCGTCTATGGGGTTCCGTTG
ATGGTTGTGAACGCGTTTCTGGTGTTGATCACTTATCTTCAACATACTCACCCTGGCTTGCCGCAATTATGATAA
AT

Figure 2. DNA sequence of *FAD2-1* gene (5'- 3).

sgRNA design

Two sgRNAs which target *FAD2-1* gene in sunflower transcribed via U3d and U6-1 promoters, were designed by CRISPOR-Tefor software. The sequences 315/fw and 497/rev had the highest specificity scores and the lowest off-target scores for 0-1-2-3-4 mismatches (Figure 3).

Position/Strand	Guide Sequence +*PAM* + Restriction Enzymes ☐ Only G- ☐ Only GG- ☐ Only A-	Specificity Score	Predicted Efficiency		Out-of-Frame score	Off-targets for 0-1-2-3-4 mismatches
			Show all Scores			+next to PAM
			Doench '16	Mor.-Mateos	Click on score to show micro-homology	
315 /fw	GCTGCGTCCTCACCGGAGTC TGG Enzymes: *HinfI, BsII, PpsI* Cloning / PCR primers	98	23	31	46	0-0-0-1-14 0-0-0-0-0 15 off-targets
497 / rev	AGTACCACGGGACTTTCGAT CGG Enzymes: *NdeII, TaqI, PcsI, Hpy188III, PvuI, Bsh1285I* Cloning / PCR primers	98	59	35	70	0-0-0-0-7 0-0-0-0-0 7 off-targets

Figure 3. The guide sequences selected by CRISPOR-Tefor.

Even though, 315/fw sequence has 1 off-target in genome with 3 mismatches, it is acceptable. The sequences were examined for GC% content in NEBcutter V2.0 and 50-70% GC pairs was accepted to ensure maximum stability. The GC% content of 497/rev is 57% and 315/fw is 70%. Additionally, the availability of restriction enzymes was checked by NEBcutter V2.0. 497/rev has target sites for *DpnI, PvuI, BsiEI, MboI, DpnII, Sau3AI, TaqI* and 315/ fw has target sites for *Bs1I, HinfI, HpaII, MspI, BsaWI, MspJI, LpnPI*. After NEBcutter analyses, the secondary structures of the sequences were examined by the mFold Web Server RNA Folding Version 2.3 Energies to eliminate the target sequences which might form hairpins or contain longer than six bp stems. The results showed that the selected sequences do not form hairpins and have no longer than six bp stems which inhibit the system (data not shown).

Vector construction and cloning

Two PCR reactions were set for each sgRNA (U3 497 and U6 315) with using UF–RP and FP–gR-R primer sets. Promoter and target regions were amplified using UF–RP primer set. Additionally, sgRNA scaffold and target regions were amplified using FP – gR-R primer set. The expected size of the product of the PCR reaction 1 (in which UF and RP primers used) was 360 bp. The band sizes obtained as expected: 135 bp for scaffold region and 360 bp for scaffold region including target (Figure 4a). The product of the second PCR was 490 bp as expected (Figure 4b).

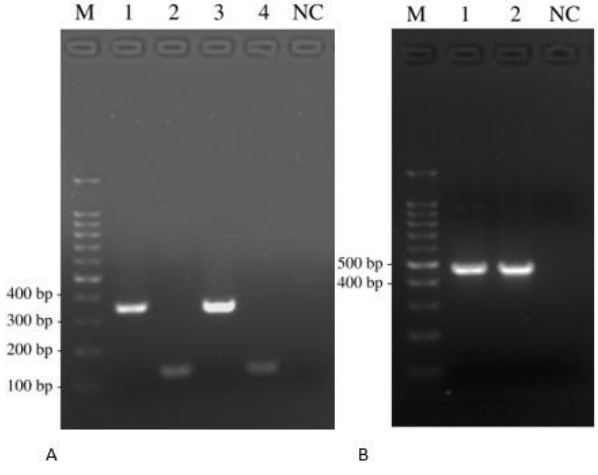

Figure 4. A. Agarose gel electrophoresis of the products of First PCR (1) AtU3d-N20 (2) N20-scaffold (U3) (3) AtU6-1-N20 (4) N20-scaffold (U6) (NC) Negative Control (M) Sizer™ - 100 bp DNA Ladder (Intron). B. Agarose gel electrophoresis of the products of Second PCR (1) U3 sgRNA expression cassette (AtU3d-N20-scaffold) (2) U6 sgRNA expression cassette (AtU6-1-N20-scaffold) (NC) Negative Control (M) Sizer™ - 100 bp DNA Ladder (Intron).

Assembly of the two sgRNA expression cassettes into the vector was carried by Gibson and Golden Gate Cloning method. For Golden Gate Cloning the sgRNA fragments were cloned in *BsaI* sites into binary vector pYLCRISPR/Cas9P35S-N by PCR. pYLCRISPR/Cas9P35S-N binary vector was derived from pCAMBIA1300 vector. Two sites having *BsaI* restriction enzyme recognition sites provide assembly of multiple sgRNAs (Chib et al., 2020). Thus, the binary vector is eligible for multiple target-sgRNAs. Targeting multiple genomic sites in a single binary vector increases the targeted mutagenesis rate (Peterson et al., 2016). Therefore, two sgRNAs targeting *FAD2-1* gene driven by the sunflower U3d and U6–1 promoter, were used to increase editing efficiency. Similarly, pYLCRISPR/Cas9 binary vector was commonly used in CRISPR Cas9 gene editing studies on different plants such as soybean (Cheng et al., 2019), saffron (Chib et al., 2020), *Medicago truncatula* Gaertn. (Zhang et al., 2020), grape (Wang et al., 2018), and cotton (Gao et al., 2017).

After the PCR reaction, the product was used directly in *E. coli* transformation. The efficiencies of Gibson and Golden Gate assembly techniques were tested and compared. pHDE-35SCas9-mCherry CRISPR/Cas9 vector, which was used in Gibson Assembly, has *PmeI* and *SpeI* restriction sites while *PmeI* restriction enzyme generates blunt ends, *SpeI* restriction enzyme generates sticky ends. Therefore, sticky and blunt ligations were required in pHDE-35SCas9-mCherry CRISPR/Cas9 vector. Since the ligation efficiency of blunt ends is much lower than the one for sticky ends, blunt ends are not preferred. However, colony PCR results after *E. coli* transformation was subjected to gel electrophoresis and no bands could be obtained with Gibson assembly. Thus, the result of gel electrophoresis indicated that sgRNA expression cassettes could not be ligated into the vector or the ligation efficiency was very low.

Differently, pYLCRISPR/Cas9 binary vector was applied in Golden Gate Assembly. The ligation product (490 bp, as expected) was observed in colony PCR (Figure 5), hence, this method was chosen for the assembly of the sgRNA expression cassettes. The vector has *BsaI* restriction sites which generate sticky ends, thereby probably increase the ligation success.

Plasmid was isolated from one colony and sequenced. The sequencing result, which was given in Figure 6, confirmed the presence of the correct sequence of cassettes in the plasmid.

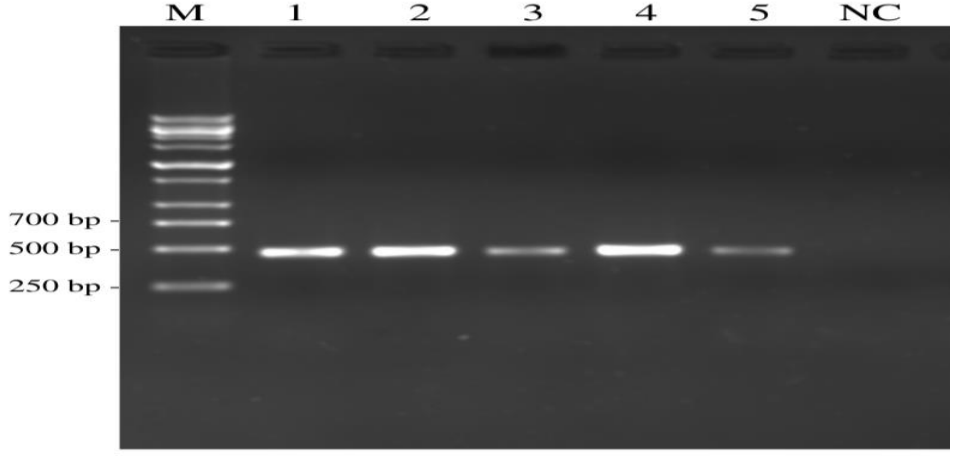

Figure 5. Agarose gel electrophoresis image of Colony PCR products (1) Sample 1 (2) Sample 2 (3) Sample 3 (4) Sample 4 (5) Sample 5 (NC) Negative Control (M) Sizer™ – 1 kb DNA Ladder (Intron).

accgATGGAATCGGCAGCAAAGGACGCGTTGACATTGTAGGACTATATTGCTCTAATAAAGGAGGCAGC
Tatgctggccgtcgttttacaacgtcgtgactgggaaaaccctggcgttacccaacttaatcgccttgc
agcacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccgatcgcccttcccaacagtt
gcgcagcctgaatggctaaataagcttatgatttctttttcttacgaattttgcgtcccacatcggta
agcgagtgaagaaataactgctttatatatggctacaaagcaccattggtcaGTACCACGGGACTTTCG
ATGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCG
AGTCGGTGCTTTTTTTCAAGAGCTTGGAGTGGATGGACCctgaCACTGGAATCGGCAGCAAAGGAAGAA
ATCTCAAAATTCCGGCAGAACAATTTTGAATCTCGATCCGTAGAAACGAGACGGTCATTGTTTTAGTTC
CACCACGATTATATTTGAAATTTACGTGAGTGTGAGTGAGACTTGCATAAGAAAATAAAATCTTTAGTT
GGGAAAAAATTCAATAATATAAATGGGCTTGAGAAGGAAGCGAGGGATAGGCCTTTTTCTAAAATAGGC
CCATTTAAGCTATTAACAATCTTCAAAAGTACCACAGCGCTTAGGTAAAGAAAGCAGCTGAGTTTATAT
ATGGTTAGAGACGAAGTAGTGATTGCTGCGTCCTCACCGGAGTCGTTTTAGAGCTAGAAATAGCAAGTT
AAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCttttttcaagagcttgg
agtggatggatcgag

Figure 6. The sequencing result of plasmid DNA. Green highlighted region represents U3 promoter sequence. Yellow highlighted region represents U6 promoter sequence. Red highlighted region represents sgRNA sequence. Turquoise highlighted region represents scaffold sequence. Pink highlighted region represents the regions where

A. tumefaciens transformation

Following the transformation, *A. tumefaciens* cells were spread on LB agar plates supplemented with adequate antibiotics: gentamycin and kanamycin for EHA105 strain and streptomycin and kanamycin for LBA4404 strain. Kanamycin is used for the selection of the vector. Additionally, gentamycin and streptomycin are used for selection of EHA105 and LBA4404 *A. tumefaciens* strains. Therefore, the transformed *A. tumefaciens* colonies were selected with these antibiotics.

Sunflower transformation

After vacuum infiltration, the half-seeds were co-cultivated with *A. tumefaciens* cells in co-cultivationmedium for two days in dark at 28°C. The survival rates of the seeds after transformation varied from 87% to 98% (Table 2). The highest survival rate was obtained in the with the co-cultivation of the half-seeds with *A. tumefaciens* EHA105 strain. Moreover, multiple shoots were obtained (1.5-1.7 shoot/explant) with 80% of rooting success (Figure 7) in SM1 and RM media, respectively. According to our preliminary studies, the cutting of the seeds was found to be an important step that had a direct impact on *in vitro* germination and the efficiency of the transformation as well (unpublished data).

The half-seeds were kept with *A. tumefaciens* culture in sterile tubes and subjected to vacuum infiltration for 30 minutes at 60 kPa as described by Sujatha et al. (2012). According to the study of Sujatha et al. (2012), vacuum infiltration was the treatment that had positive effect on transformation efficiency and 30 minutes were reported as the optimal time period of the treatment. Therefore, vacuum infiltration was applied for 30 minutes to enhance agro infection in this study.

Table 2. Co-cultivation results of transformation and control half-seeds

Co-cultivation	First Trial			Second Trial		
Experiment Groups	**Number of Plant Material**	**Survival**	**Survival%**	**Number of Plant Material**	**Survival**	**Survival%**
Deray – LBA4404	100	87	87%	50	46	92%
2453A - LBA4404	100	91	91%	50	48	96%
Deray – EHA105	100	83	83%	50	48	96%
2453A - EHA105	100	89	89%	50	49	98%
Deray – Control	50	46	92%	50	45	90%
2453A - Control	50	47	94%	50	47	94%

Figure 7. *In vitro* shoot (left) and root (right) formation of transformed sunflower in kanamycin containing selection media.

Analysis of putative transformed shoots by PCR

PCR results of *FAD2-1* (Figure 8) and kanamycin (Figure 9) genes demonstrated that both genes were successfully integrated in putative transformants. Moreover, PCR product was obtained with Pps F1 ve Pgs R2 primers as they amplified the T-DNA region while no band was seen with PmeI and Pgs R2 primers since PmeI primer amplified the outer region of T-DNA in the pYLCRISPR vector (data not shown).

This result showed that T-DNA (including the *FAD2-1* and kanamycin genes) was successfully transformed to sunflower and the presence of the PCR product was not resulted from *A. tumefaciens* contamination since no PCR product was obtained with PmeI and PgsR2 primers.

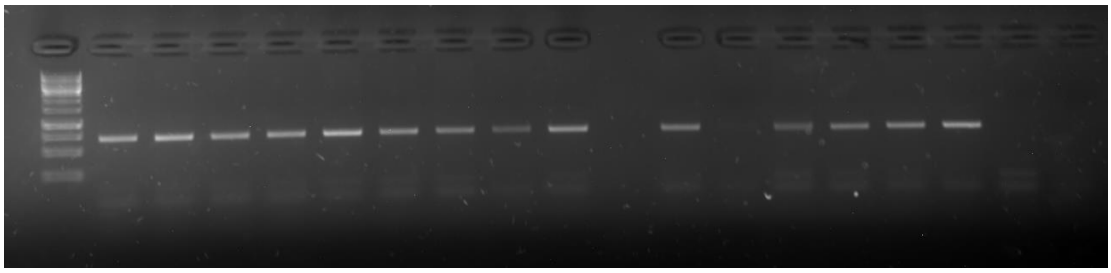

Figure 8. PCR result of *FAD2-1* gene. 1. Lane Marker, 2-16 lanes putative transformants, 17 and 18. lanes negative control.

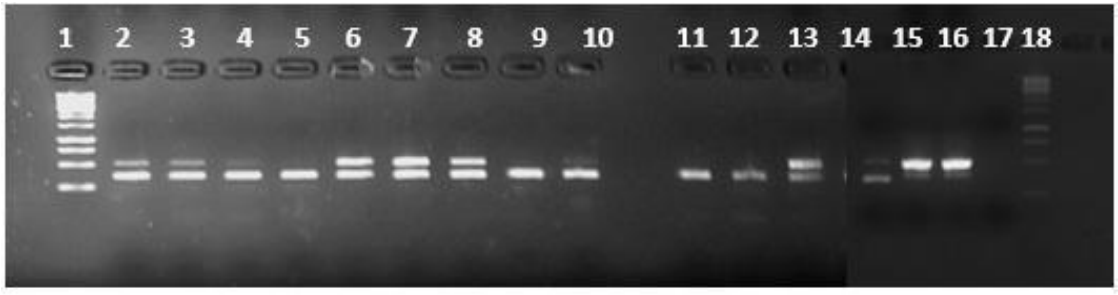

Figure 9. PCR result of kanamycin gene. 1. and 18 Lanes Marker, 2-16 lanes putative transformants, 17. lane negative control.

Conclusion

According to our results, sequencing of PCR product of *FAD2-1* gene confirmed absence of mutations. Although the transformation rates were high in both sunflower genotypes, the absence of mutations in the gene sequence of PCR product of *FAD2-1* gene indicated that the vector transferred to sunflower did not work properly. Previous studies reported the failure rate of the CRISPR gene editing system was found to be 15%. Possibly the reason of failure of CRISPR/Cas9 system might be due to the permanent attachment of Cas9 protein to the relevant region and thus DNA repair enzymes cannot reach the double strand break and also presence of methylated P35S promoter region may be the another reason for ineffective promoter as well. Hence, the efficieny of CRISPR/Cas9 could be increased with the assessment of different promoters other than 35S promoter in sunflower.

Acknowledgments

We gratefully acknowledge the support of the Marmara University Research Foundation BAPKO (Project No: FEN-C-YLP-120619-0193) for their financial support.

Conflict of interest

Authors declare no conflict of interest.

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