FAD2-1 RNAI CONSTRUCT DESIGN FOR GENE SILENCING IN *JATROPHA CURCAS* TO INCREASE LEVEL OF SEED OLEIC ACID

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Abstract

Jatropha (*Jatropha curcas*) is a promising energy crop due to the high amount of seed oil that can be converted into biodiesel. A high-quality biodiesel contains less polyunsaturated fatty acids and more monounsaturated fatty acids. Jatropha seed oil has 30 to 50% polyunsaturated fatty acids (mainly linoleic acid), that negatively affectsits quality and performance. The enzyme 1-acyl-2-oleoyl-sn-glycero-3-phosphocholine Δ12-desaturase (*FAD2*) is the key enzyme for the production of linoleic acid. The aim of the study isdownregulating the expression of Δ12 fatty acid desaturase gene in *Jatroha curcas* (*JcFAD2-1*gene) that encodes the enzymeresponsible of conversion of oleic acid into linoleic acidby RNA interference (RNAi) technology to block its action. To achieve the objective, RNAi induced vectortargeting the *FAD2-1* gene was designed. The *FAD2-1* RNAi construct is ready to be used for further transformation experiments. The represented protocol could be successfully applied to increase the percentage of oleic acid versus linoleic acid in *Jatroha curcas* seed oil through genetic engineering for enhancing the oil quality.

Key words: Jatropha, *FAD2-1*, RNAi, gene expression downregulation, seed oil, biodiesel.

Introduction

Biofuel is a renewable alternative to fossil fuel to block the gap between over population and unrenewable fuel depletion. Biodiesel is one of the most common biofuel types of increasing demand. Its properties are highly dependent on the composition of fatty acids in the oil. The biodiesel derived from plant oil are generally composed of five common fatty acids: palmitate, stearate, oleate, linoleate and linolenate. Palmitate and stearate are saturated fatty acids, oleate is a monounsaturated fatty acid, while linoleate and linolenate are polyunsaturated fatty acids (Qu *et al*., 2012). Biodiesel with high content of monounsaturated fatty acid (oleate) has excellent characteristics regarding ignition quality, nitrogen oxides (NOx) emissions and fuel stability. However, most oils derived from plant and used as biodiesel have a high level of polyunsaturated fatty acids (linoleate and linolenate acids), which negatively affects the biodiesel quality by decreasingits stability and affecting

the cetane number (CN). The value of CN is a determinant factor for the ignition quality of diesel fuels by decreasing NOx emissions (Qu *et al*., 2012 and Li *et al*., 2016).

Attention has been given to plant species that produce non-edible oils, such as Jatropha (*Jatropha curcas* L.), which is drought tolerant and cangrow on degraded soils and waste lands. Ithas high seed yield and seed oil content, which is a promising renewable biodiesel alternative to conventional one (Wassner *et al*., 2016). Jatrophaseeds contain about 25-40% oil by weight, 20% saturated fatty acids and 80% unsaturated fatty acids (Venkatachalam *et al*., 2019). Seed oil contains 30 to 50% poly unsaturated fatty acids (mainly linoleic acid), therefore, there is a need to improve its fatty acid profile (Qu *et al*., 2012). Clemente and Cahoon (2009) reported that producing plants with high oleic acid level by conventional breeding is not easy for the difficulties in finding a suitable germplasm. Selection of natural variants or induced mutants with high oleic acid level for traditional

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breeding is time-consuming and is limited by the available genetic resources and mutants with increased oleic acid content are often associated with undesired agronomic properties.

Alternatively, genetic engineering technology can be used to increase oleic acid content in Jatropha oil. In *Jatrophacurcas*, ∆12 fatty acid desaturase (*JcFAD2-1*) gene mediates the conversion of oleic acid to linoleic acid. By blocking its expression using RNA interference (RNAi), it is possible to generate plants with increased level of oleic acid and an estimated optimum CN as high as 60.2 (Qu *et al*., 2012).

Gene silencing using RNAi technique, for the manipulation and increment of fatty acid composition of oil, was reported to be more effective than other silencing techniques, showing higher degree of silencing and frequency of transgenic plants. It has been successfully applied in various oilseed crops; such assoybean (Mroczka *et al*., 2010 and Wagner *et al*., 2010), rice (Zaplin *et al*., 2013) and flax (Chen *et al*., 2015).

In respect to oil improving in *Jatroha curcas*, *JcFAD2-1* RNAi was applied for the first time to improve its agronomic traits and seed oil quality (Qu *et al*., 2012). Qu *et al.,* (2012) used the chemical inducible Crelox system to obtain marker-free transgenic Jatropha to improve its agronomic traits and seed oil quality. The construction of transformation vectors for *FAD2* gene silencing was designed using inverted repeat (IR) and intron-spliced inverted repeat (ISIR) orientations to generate hair-pin RNAs (hpRNA), to modify fatty acid composition, especially increasing oleic acid and reduction linoleic acid content of Jatropha oil (Utomo *et al*., 2015).

FAD2 is the key enzyme responsible for the biosynthesis of polyunsaturated fatty acids in non-photosynthetic tissues of oilseed plants (Dar *et al*., 2017). Improving biodiesel quality from a non-edible sustainable source as Jatropha seed oil is of great importance. Fewresearches were performed in this field and the increasing global interest for environment and human health saving, necessitate more research for biodiesel enhancement.

The aim of the study is downregulating the expression of *JcFAD2-1* gene, responsible of conversion of oleic acid into linoleic acid in Jatropha oil by RNA interference technology to block its action. The construction of transformed vector for down regulated *FAD2-1* gene is the first step to modify fatty acid composition of *Jatroha curcas* seed oil for enhancing the oil quality.

Materials and Methods

FAD2-1 gene fragment amplification and cloning

First strand cDNA was synthesized from total RNA of *Jatropha curcas* leaves using Super Script™ III M-MLV Reverse Transcriptase (Invitrogen, USA) according to them anufacturer's instructions. A partial sequence of *FAD2* gene from *Jatropha curcas* was amplified using degenerate primers and cDNA as a template. Primers were designed based on the sequence information available in National Center for Biotechnology Information; NCBI GenBank (http://ncbi.nlm.nih.gov/genbank). The degenerate primers sequence was based on the conserved region of the coding sequence of *FAD2* gene from other plant species. The primer pairs; *JcFAD2-*1FD1, 5´-ATGGGDGSDGGHGGMMGMA-3´ and JcFAD2-1RD276, 5´CCARTARABNGGCCANGCN AHG-3´were selected to amplify the 270 bp *FAD2* fragment. According to the sequencing results, the amplified fragment isa partial sequence of *J. curcas FAD2-1* (*JcFAD2-1*) gene. Therefore, to construct *FAD2-1* RNAi cassette, an other two specific primersets (Table 1) were designed to amplify sense and antisense oriented fragments between 9980 and 10249 nts of *JcFAD2-1*gene. The amplification reaction was carried out by denaturing the template DNA at 95°C for 4 minutes, followed by 25 cycles heating at 95°C for 30 seconds, annealing at 53.5ºC for 30 seconds and extension at 72°C for 30 seconds and the reaction was ended by 7 minutes at 72°C. A 121 bp derived from the 5'UTR (861 and 982 nts) of *JcFAD2-1* upstream the coding sequence was amplified using genomic DNA as a template. The primers used in the amplification and their restriction sequences that flanked each one are represented in table 1.

The amplified PCR products were purified using Gene JET PCR Purification Kit (Thermoscientific, USA), digested with appropriated restriction enzymes and then cloned one by one into p Blue Script SK+ (pSK+) cloning vector using T4 ligase (NEB, UK). The ligated DNA was transformed into *Escherichia coli* bacterium strain DH10² chemically competent cells (Invitrogen). Following bacterial overnight growth and plasmid DNA purification, the cloned fragments were subjected to sequence analysis by the facility of Macrogen Korea.

FAD2-1 RNAi plant expression construct

The assembled RNAi cassette, containing sense and antisense oriented *FAD2-1* fragments and *FAD2-1* intron located in between the two fragments, was released from pSK+ vector by restriction digestion using *Nde*I/*Sac*I enzymes. The DNA band was gel purified and subcloned into the corresponding restriction sites of pRI201 plant expression vector (Takara, Japan). The positive clones were screened by PCR reaction using pRI-specific primer set (Table 1).

Table 1: Specific primers and restriction enzymes used in PCR and dsRNA synthesis.

Primer Name	Sequence (5' - 3')	Restriction enzyme
JcFAD2-1sense FD	AACC<u>GTCGAC</u>C<u>ATATG</u>ATGGGTGCCGGTGGCAGAATG	*Sal*I, *Nde*I
JcFAD2-1sense RD	AACC<u>AAGCTT</u>CCAGTAAATTGGCCAGGCCAC	*Hind*III
JcFAD2-1antisense FD	TTGG<u>TCTAGA</u>CCAGTAAATTGGCCAGGCCAC	*Xba*I
JcFAD2-1antisense RD	GGCC<u>GAGCTC</u>ATGGGTGCCGGTGGCAGAATG	*Sac*I
JcFAD2-1intron FD	AACC<u>AAGCTT</u>CACGTGTATGTGCAAACACGTG	*Hind*III
JcFAD2-1intron RD	TTGG<u>TCTAGA</u>CGGCTACATCCATTCGTCCATG	*Xba*I
pRI FD	GCGCGCGCGGCCGCGCCTGCAGGTGCCCAG	
pRI RD	GCGCGCCCCGGGTTCATTCCATAGTCC	

Introducing *FAD2-1* RNAi cassette into *Agrobacterium tumefaciens*

The pRI201 carrying *FAD2-1*-specific RNAi cassette was introduced into *Agrobacteriumtumefaciens* LB4404 strain. Positive transformation of *Agrobacterium tumefaciens* bacterial colonies was checked for vector harboring cells using colony PCR by JcFAD2-1sense FD/ JcFAD2-1sense RD and pRI FD/pRI RD primers. Putative positive clones were grown overnight on LB medium containing kanamycin and was confirmed by restriction analysis. Positive bacterial colonies were glycerol stocked and preserved at-80°C for transformation experiments into *Jatrophacurcas* plant.

Results and Discussion

The first strand cDNA, prepared from total RNA of *Jatropha curcas* leaves, was used as a template for the amplification of partial fragment of *FAD2-1* gene. Qu *et al*., (2012) identified three *FAD2* genes from *Jatrophacurcas*, therefore, a degenerate primer set was decided to be used to isolate one of these genes or any other new iso form, if any, of *JcFAD2* gene. One degenerate primer set was designed from the conserved regions of *JcFAD2* gene in other plant species; *Carthamustictorius, Camelinasativa*, *Hiptagebeneghalensis, Brassicanapus*, *Glycinemax* and *Helianthusannus*. A 270 bp fragment was amplified using the degenerate primers (Fig. 1). The identity of the fragment was verified by BLASTX and BLASTN on the GenBank database. The identified sequence showed 100% match with position 9980-10249 nts of *JsFAD2-1* gene (Accession number JN544421.1).

The cloning strategy of dsRNA-producing cassette within pRI201 plant expression vector is summarized in two major steps. Firstly, the dual DNA fragment of *FAD2-1* was double cloned in opposite directions in respect to each other and flanked with stuffer sequence, derived

Fig. 1: PCR amplified 270 bp *JcFAD2-1* fragment using JcFAD2-1FD1/JcFAD2-1RD276de generate primer set and cDNA as a template.
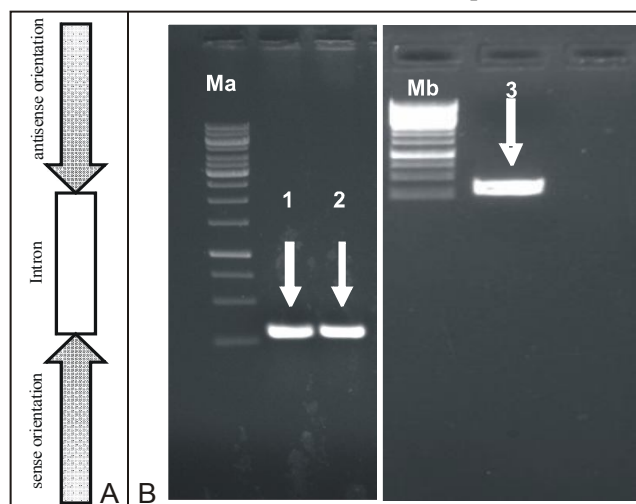

Fig. 2: (A) Map of assembled cassette to be cloned into the pBuescrtip SK+ vector. (B) PCR amplified sense, antisense and intron fragments of *JcFAD2-1* gene from *Jatropha curcas*; (1) 270 bp sense fragment using specific primer set (JcFAD2-1 sense FD/JcFAD2-1 sense RD), (2) 270 bp antisense fragment using specific primer set (JcFAD2-1antisense FD/JcFAD2-1 antisense RD) and (3) 130 bp intron using specific primer set (JcFAD2-1 intron FD/JcFAD2-1 intron RD). DNA marker;1 kb (Ma) and 50 bp (Mb).

from the *FAD2-1* intron sequence between 861-982nts, in between them with in pBluescript SK+ vector. Secondly, the assembled DNA fragment was released and further subcloned into the multiple cloning site of pRI201 vector downstream the Cauliflower mosaic virus (CaMV 35S) promotor and upstream the *Arabidopsis* heat shock protein (HSP) terminator signal using NdeI and SacI restriction enzymes.

To construct a dsRNA-producing cassette (Fig. 2A), the 270 bp *FAD2-1* fragment was amplified using JcFAD2-1 sense specific primer set that is modified with *Sal*I/*Nde*I restriction sites on the 5'-end and *Hind* III site on the 3'end (Fig. 2B-1). Secondly, 121bp intron fragment was amplified using *JcFAD2-1* intron specific primer set (Fig. 2B-3) that carrying *Hind* III and *Xba* I restriction sites on the 5' and 3' -ends, respectively. Finally, the 270bp fragment was amplified using *JcFAD2-1* antisense specific primer set with *Xba* I and *Sac* I restriction sites on the 5' and 3'-ends, respectively (Fig. 2B-2). Basically, sense and antisense primer sets have the same nucleotide sequence, however, they were designed to facing each other during cloning.

Double digestion reactions of amplified fragments and corresponding vectors are shown in fig. 3. The pSK+ cloning vector was linearized using *Sal* I/*Hind* III restriction enzymes (Fig. 3B). Thesense oriented *FAD2-1* fragment was double digested with the same restriction enzymes (Fig. 3A) and was ligated into linearized pSK+ forming pSKF. Both intron fragment and pSKF were double digested with *Hind* III/*Xba* I restriction enzymes (Fig. 3C and D). Digested intron and plasmid were overnight ligated forming pSKFI. A *Xba* I/*Sac* I digested antisense oriented *FAD2-1* fragment (Fig. 3E) was ligated
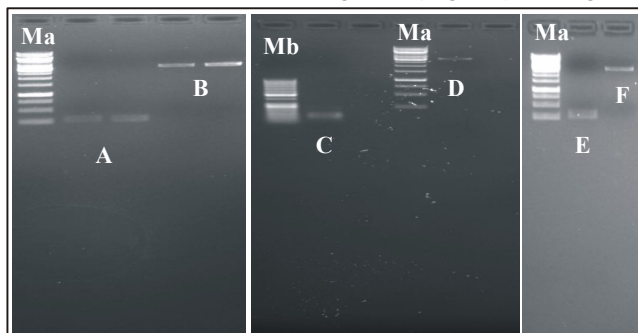

Fig. 4: Confirmational analysis of positive clones; (A) PCR products of expected size at 661 bp and clone 10 was selected for next step. (B) Restriction digestion of clone 10 using *Sal* I/*Sac* I enzymes released dsRNA-producing fragment (pointed by arrows) and pSK+ vector (marked by strikes).

into the corresponding sites of pSKFI (Fig. 3F) creating pSKFIAn construct.

PCR and restriction analysis revealed positive cloning of dsRNA-producing cassette into pSK+ cloning vector. The putative positive clones were selected for PCR confirmation analysis using M13 universal primer set. The PCR products of expected size are shown in fig. 4A. Clone 10 was selected for further restriction enzyme analysis using *Sal* I/*Sac* I enzymes. The digestion reactions produced DNA fragment of expected size at 661 bp (Fig. 4B).

Nucleotide sequence of 661 bps dsRNA producing fragment was confirmed by DNA sequencing and then cloned into pRI201 plant expression vector. A 661 bp fragment was released from pSKFIA ncon struct using *Nde* I/*Sac* I restriction enzymes and ligated to the corresponding sites of the linearized pRI201 vector. The

Fig. 3: Restriction enzyme digestion of *FAD2-1* fragments, intron and pSK+ cloning vector. (1) Double digestion of sense oriented *FAD2-1* fragment (A) and pSK+(B) using *Sal* I/*Hind* III. (2) Intron fragment (C) and pSKFI construct (D) are double digested with *Hind* III/*Xba* I. (3) Antisense oriented *FAD2-1* fragmentwas double digested using *Xba* I/*Sac* I (E) and pSKFI was digested with the same enzymes (F). DNA marker; 1 kb (Ma) and 50 bp (Mb).
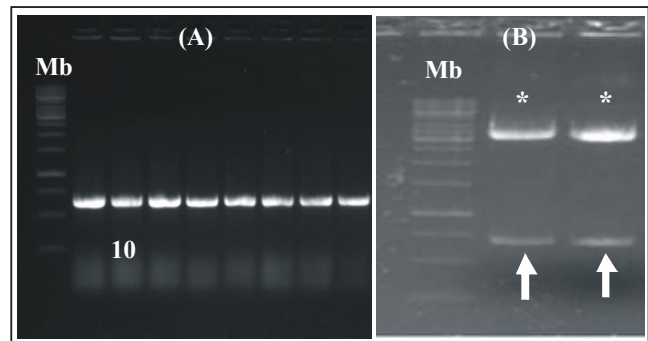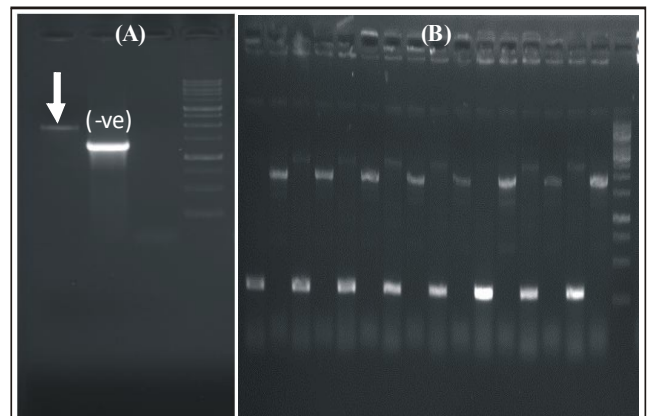

Fig. 5: (A) PCR analysis of positive cloning of *FAD2-1* dsRNA producing cassette into pRI201 vector; (-ve) is negative control and positive colony is marked with white arrow. (B) Colony PCR sceening of *Agrobacteriumtumefaciens* cells using JcFAD2-1sense FD/JcFAD2-1 sense RD and pRI FD/pRI RD primers.

positive clones were confirmed using PCR, the negative control PCR reaction results in 1111bp fragment, while positive cloning produced 1772bp band (Fig. 5A). The pRI201 construct carrying dsRNA-producing cassette (pRI201-FADRNAi) was transformed into *Agrobacteriumtumefaciens* LB4404 strain. The positive colonies for LB4404 cells were confirmed using *Nde* I/*Sac* I restriction enzymes showing expected size of 661bp (Fig. 5B).

RNAi is a post-translation gene silencing technique that is used to study gene function, also it has been successfully used for significantly down-regulating the target gene in a number of plant species including *Jatropha curcas* (Yin *et al*., 2010 and Palle *et al*., 2013). More than 98% reduction in the accumulation of curcin transcript in the Jatropha leaf tissues was reported (Patade *et al*., 2014). Curcin, encoded by curcin precursor gene, is a phytotoxin present in Jatropha hindering its usage as animal feed after oil extraction. Knock down the curcin gene expression was achieved by transgenic Jatropha expressing dsRNA targets curcin precursor. A promising industrial application of Jatropha is the production of biodiesel fuel. However, fuel with high content of oleic acid has excellent characteristics regarding ignition quality, NOx emissions and stability. The results obtained by Qu *et al.,* (2010) showed that *FAD2-1* is the key enzyme responsible for conversion of oleic acid to linoleic acid in Jatropha. Improving the fatty acid profile of Jatropha plants with high oleic acid level could be achieved by down regulating the *FAD2-1* expression (Gu *et al*., 2012 and Qu *et al*., 2012). Qu *et al.,* (2012) used the chemical inducible Crelox system to produce marker-free transgenic Jatropha with high oleic acid content seeds. A chemically-regulated seed-specific *JcFAD2-1* RNAi transgenic lines were generated using cassette targeting *FAD2-1* coding regionbetween 85 to 946 nts. The transgenic lines were firstly generated by conventional process usinghygromycin phosphotransferase (HPT) as selectable marker, subsequently, it was removed from the plant genome by chemically regulated DNA excision using Crelox mediated system. The resulting transgenic plants showed a dramatic increase of oleic acid (>78%) and a corresponding reduction in polyunsaturated fatty acids (<3%) in its seed oil (Qu *et al.*, 2012). In another study, two transformation vectors constructed to target *FAD2* gene. One vector was designed using IR by two unequal fragments facing each other; sense orientation of 500 nts fragment while antisense oriented fragment with 700 nts. The other vector was designed with the same length fragments of 500nts and spaced by ISIR to generate hpRNA (Utomo *et al*., 2015).

In the present study, designing of RNAi construct is reported to be used for genetic transformation of *Jatropha curcas* to downregulate the *FAD2-1* transcript expression and in turn increase the oleic acid content in seed oil. Abinary silencing vector is designed by cloning part of *FAD2-1* precursor gene in sense and antisense orientations, separated by intron. The dsRNA producing cassette targeting *FAD2-1* coding region is between 9980 and 10249nts. The two *FAD2-1* fragments were spaced with 121bp intron to serve as hairpin loop during the formation of dsRNA within the plant cells. The hpRNA has an efficient silencing effect in a wide range of plant species in constructs containing sense/anti-sense arms ranging from 98 to 853 nts (Wesley *et al*., 2001). Further, inclusion of an intron in these constructs (hpRNA) had a consistently enhancing silencing effect (Wesley *et al*., 2001). At the molecular level, systematic degradation of specific mRNA by small interfering (siRNA) between 20 to 25 nts is produced by excision of dsRNA by genetic machinery of the host cell.

RNA interference (RNAi) pathway to silence *FAD2-1* precursor gene holds huge promise for industrial applications of Jatropha. *FAD2-1*RNAi cassette is expected to dramatically increase the level of oleic acid versus linoleic acid in Jatropha seed oil, enhancing the oil quality. The construct resulted from the current work is being usedfor genetic transformation experiments to generate RNAi transgenic lines of Jatropha plants.

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