Transformation of oil palm calli using CRISPR/Cas9 System: toward genome editing of oil palm

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Abstract. Genome editing using CRISPR/Cas9 construct containing sgRNA is the newest technology used to assemble organisms with desired characters through knock out of the target gene. As part of the effort to develop oil palm planting material tolerant to Ganoderma, this research aimed to obtain a technique for introducing CRISPR/Cas9 modules containing sgRNA into oil palm tissue. Based on differential expression studies, two genes encoding isoflavone reductase (IFR) and methallothionine-like protein (MT) were selected as the target for edited genes. The CRISPR-IFR and CRISPR-MT modules each carrying a sequence of gene recognition (sgRNA) were constructed and transformed into oil palm calli using Agrobacterium tumefaciens. The transformed calli were cultured on a modified DF medium for callus propagation, and then sub-cultured into DF media for induction of somatic embryos. PCR analyses using specific primers flanking the DNA targeted region were carried out and the results showed that both constructs had been introduced into the cells. DNA sequence analysis indicated the presence of substitution of several bases in the target area of both genes. The transformed calli grew in a selection medium containing hygromycin. Development of somatic embryos was detected after 3-4 weeks culturing on modified DF media.

1. Introduction

Basal Stem Rot (BSR) disease caused by Ganoderma sp. has greatly threatened the sustainability of oil palm plantations especially in Indonesia and Malaysia [1]. The infected plants are usually dead; consequently, decrease the number of plants per unit area which in turn decrease the productivity and cause huge losses. At an average attack rate of 1%, the value of losses could reach 256 million USD per year [2]. Many efforts have been conducted to overcome this disease; unfortunately, the decline of Ganoderma attack has not been seen. On the contrary, the number of affected plants is reported to be increasing year to year. The use of planting material tolerant to Ganoderma is believed to be the most potential approaches to overcome this problem.

Cisgenesis, one of the modern biotechnology-based approaches, is claimed to be a potential technology for creating new varieties of plant in relatively quick and precise manner. Since the cassette of DNA used for modifying the targeted genome is derived from the same plant, the modified targeted plant is not categorized as a transgenic plant [3]. Genome editing, one of cisgenic technology,
is the most prospective technology to replace random mutagenesis methods, such as ethyl methanesulfonate (EMS) mutagenesis. Genome editing allows proper genetic manipulation of specific genome sequences [4, 5]. The ideal scenario of genome editing is to minimize insertion of the non-crop genetic sequences into host plant genomes by cutting out the entire non-crop DNA sequences such as gene markers for selection, Cas proteins, etc., after the target gene edited [6].

There are three modules of DNA cleavage, (1) Zinc Finger Nucleases (ZFNs), (2) Transcription Activator-Like Effector Nucleases (TALENs) and (3) The immune system adaptive prokaryotic type II CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR-associated) [4, 7, 8]. Both nucleases (ZFNs and TALENs) are used to mutate the plant genome at the specific locus. However, these systems have limited applications because they require two different DNA protein binding and flanking sequences, each with a module nuclease FoK1 [4, 9]. Compared to ZFNs and TALENs, the CRISPR/Cas9 module is simpler and considered as a breakthrough technique for genome editing. The module is based on Cas9 nuclease and a modified RNA single guide (sgRNA) to edit target nucleotide sequences [10]. These systems rely on sequence-specific nucleases (SSNs) to produce double-strand breaks (DSBs) at specific locations within the genome. DBS is repaired by the cell using a mechanism of non-homologous end joining (NHEJ) or homologous repair (HR) [9, 11].

Applications of CRISPR/Cas9 system have been reported in several plant species such as Arabidopsis thaliana, Nicotiana benthamiana, Oryza sativa, Triticum aestivum, Solanum lycopersicum, and Sorghum bicolor [4, 9]. By the end of 2014, genome editing has also been successfully applied on citrus and tomato plants [12, 13]. So far, there was no report on the application of CRISPR/Cas9 on genome editing of oil palm. As a part of the effort to develop oil palm planting material tolerant to Ganoderma through genome editing, this research aims to establish technique for delivering CRISPR/Cas9 module containing sgRNA for the model genes, namely metallothionein-like protein (MT) and isoflavone reductase (IFR) into oil palm calli and to regenerate the transformed calli into somatic embryos.

2. Materials and Methods

2.1. Callus induction from oil palm young leaf explant

Young leaf pieces were cultured on MS modified solid medium containing auxin and cytokinin for callus induction according to the method described by Sumaryono et al. [14]. The culture was then incubated in a dark room at 26°C. Initial callus began to appear after 4 to 6 months. Callus proliferation was then conducted by sub culturing every 8 weeks on the same medium with a lower auxin concentration. This callus was then used for transformation using the CRISPR/Cas9 module.

2.2. Construction of CRISPR/Cas9 system with targeted model genes on oil palm

Two genes were selected as a target for genome editing, namely isoflavone reductase (IFR) and metallothioneine like protein (MT). Construction of CRISPR/Cas9 system specifically targeted for each gene was initiated by determining the target site (also known as protospacer). From the site, 20 nucleotides were selected following the criteria of Hwang et al. [15] for designing primer of the target sequence. The design of single guide RNA (sgRNA) and Cas9 proteins was conducted using Geneious software following modified protocol of Ran et al. [16]. If the target DNA had more than one isoform with sequence differences, chimeric guide RNAs (sgRNAs) would be designed to target 1-2 gene model sites.
Table 1. Primers used in this experiment with its DNA sequences.

| Primer Name         | DNA Sequences          |
|---------------------|------------------------|
| CRISPR-F            | 5’- GACCAAGCCCGTTATTCTGAC-3’ |
| CRISPR-R            | 5’- AAGTCTGATGCAGAAGCGAG-3’ |
| NPTII-F             | 5’- GAAAGAATGGATTGACGC-3’ |
| NPTII-R             | 5’- GAAGAACTCGTCAAGAAGGC-3’ |
| HYG-F               | 5’- ACTATCGCGAGTACTTCTACA-3’ |
| HYG-R               | 5’- GTATCACTGGAAACTTGATG-3’ |
| sgRNA1 (IFR)        | 5’-GTCGAACGTGTACCTGATCtgg-3’ |
| sgRNA2 (MT)         | 5’-GAAGCTCCTGGACGACTTCAg-ag-3’ |
| Isoflavone reductase II (EgIFR-F2) | 5’-ACCGGGTACATCGGAAGTT-3’ |
| Isoflavone reductase II (EgIFR-R2) | 5’-GGAGATCACCGTGACTCCCTAC-3’ |
| Isoflavone reductase I (EgIFR-F1) | 5’-GATCATCGCCGCAATAAAAG-3’ |
| Isoflavone reductase I (EgIFR-R1) | 5’-ATGGGAGGAAGTAACCAGCA-3’ |
| Metallothionein-like protein I (EgMT-F1) | 5’-AGGCAAATGTGGCTGTGGCGTT-3’ |
| Metallothionein-like protein I (EgMT-R1) | 5’-ACTTGCAAGTGGACGCTCCCTA-3’ |
| Metallothionein-like protein II (EgMT-F2) | 5’-GGGCACATGAAGGGTTGGA-3’ |
| Metallothionein-like protein II (EgMT-R2) | 5’-TTGGATGCTTGGAAGGAGAC-3’ |

2.3. Establishment of transformation methods using Agrobacterium tumefaciens carrying CRISPR/Cas9 module

DNA transformation of oil palm callus was carried out using A. tumefaciens system carrying the CRISPR/Cas9 constructs, each with sgRNA specifically targeted for MT or IFR genes (CRISPR-MT and CRISPR-IFR). Each construct was first introduced into A. tumefaciens. In addition, both constructs were also cloned into E. coli. The transformation process was done by immersing the oil palm callus along with A. tumefaciens for 15 min. The callus was then cultured on the co-cultivation media containing 100 ppm acetosiringone for two days followed by culture on media containing 250 ppm cefotaxime for 5 days. The calli were then transferred into selection media containing 25 ppm hygromycin and 250 ppm cefotaxime for 3-4 weeks. The transformed calli were cultured on the media for callus multiplication. Successful introduction of CRISPR-MT and CRISPR-IFR into A. tumefaciens and E. coli was confirmed by PCR using NPTII primers, HYG primers and CRISPR monocot-specific primers, followed by DNA analysis. The callus was then sub cultured into the media for regeneration of somatic embryos. DNA sequences of the primers used in this research were shown in Table 1.

3. Results and Discussion

3.1. Introduction of CRISPR constructs into A. tumefaciens and E. coli

Figures 1 and 2 illustrate the electrophoresis of colony PCR products of E. coli and A. tumefaciens transformed using CRISPR-MT and CRISPR-IFR constructs. These results showed that both constructs have been introduced into A. tumefaciens and cloned into E. coli. The A. tumefaciens containing CRISPR constructs with sgRNA of MT and IFR genes were then used in the transformation of oil palm calli for editing MT and IFR genes.
Figure 1. (A) Colony PCR using NPTII primers of the transformed *E. coli*. Lanes (1-9) colonies transformed using CRISPR-MLP: (10) Control pCAMBIA 1303 P5CS, (11) negative control, (12-16) colonies transformed with CRISPR-IFR. (B) Colony PCR using CRISPR primers: lanes (1-6) colonies transformed with CRISPR-MT, (7) plasmid DNA CRISPR-MT, (8) negative control, (9) plasmid DNA CRISPR-IFR, (10-15) colonies transformed with CRISPR-IFR, (M) Marker 1 kb plus ladder.

Figure 2. (A) Colony PCR using NPTII primer of *A. tumefaciens* transformed using CRISPR modules. Lanes (1-7) colonies transformed by CRISPR-IFR construct, (8) Control pCAMBIA 1303 P5CS, (9) Negative control, (10-13) colonies transformed by CRISPR-MT construct, (B) Colony PCR of *A. tumefaciens* using CRISPR primer. (1-4): colonies transformed with CRISPR-IFR construct, (5) plasmid DNA of CRISPR-IFR, (6) negative control, (7) control of Plasmid DNA LBA4404, (8) plasmid DNA of CRISPR-MT, (9-12) colonies transformed with CRISPR-MT construct, (M) 1 kb plus DNA ladder.

3.2. Transformation of oil palm calli using CRISPR-IFL and CRISPR-MT constructs

Development of oil palm calli transformed with both CRISPR/Cas9 constructs into somatic embryos is presented in Figure 3. It is shown that on the media containing hygromycin as selected marker, callus transformed with CRISPR construct developed into somatic embryos (Figure 3E and 3F). On contrary, the untransformed calli did not develop and tend to browning. This result, followed by PCR analysis using specific genes (IFR and MT) and hygromycin primers prove that both constructs have been introduced into oil palm genome (Figure 4).

To identify the occurrence of mutation on the sgRNA targeted DNA region, the DNA sequences of these PCR products were aligned with that of the control untransformed DNA. Figure 5 show that introduction of CRISPR-IFR2 construct (A1-A6) induce InDels mutation causing changes of amino acid sequences which may alter the function of its proteins. It can be seen on A3, A4 and A5 lines that there is nucleotide substitution, resulted in CAGCTT ---GG-AA-T sequence compared with the untransformed control, AGCT CC---AC-CT-C-A. In those three lines, deletion was also occurred in the 111 to 115 nucleotide sequences. On the other side, there is no mutation detected on the A1, A2, and A6 lines. When viewed from the protein translation, substitutions on A3, 4 and 5 change the sequence of amino acid residues, i.e. TALGEIRVRV compared with the untransformed control, KLLDDFKASGV. Changes the sequence of amino acid residues is confirmed to have an effect on the function of the IFR2 protein, however further proof has to be observed on the developing transformed callus.
Figure 3. Oil palm calli 5 days after transformation using CRISPR-IFR (A) and using CRISPR-MT (B), untransformed callus on the media without antibiotic (control) (C), untransformed callus on the media with antibiotic (negative control) (D), somatic embryo developed from the CRISPR-IFR–transformed callus (E), somatic embryo developed from the CRISPR-MT–transformed callus (F), and somatic embryo developed from untransformed callus on the media without antibiotic (positive control) (G).

Figure 4. PCR of oil palm calli using primer of MT2 (1-6) dan MT1 (7-13) (A). Lanes 1, 7: negative control, 2, 8, & 10: untransformed calli, 3 & 9 plasmid DNA of CRISPR-MT, 4-6, & 11-13 calli transformed by CRISPR-MT; using IFR2 primers (B). Lane 1: Negative control, 2: untransformed calli, 3 & 4 calli transformed by CRISPR-IFR; hygromycin primers (C). Lane 1: untransformed callus, 2: negative control, 3 & 4 calli transformed by CRISPR-IFR, 5 - 7: calli transformed by CRISPR-MT; (M) 1 kb plus DNA ladder.

DNA alignment of the sgRNA target region of the transformed oil palm calli using CRISPR-MT constructs showed that introduction of CRISPR-MT construct induced mutation in the sgRNA region which may cause the MT protein to no longer function. DNA alignment is carried out between untransformed callus (NT-MT) and transformed callus (B1, 7, 9, and 10) which may be considered as a new strain. It can be seen in Figure 6 that the most basic substitution occurs in the B9 strain with the change of GAT-A-ATA-T------T---CC which converts the translation of the protein into IKINVYSALG compared with the untransformed control, RCNVYPDLGG. Individual substitution occurs in the B7 strain that changes the residual amino acid N (on the untransformed calli/control) to G. The basic substitution in line B10 converts the amino acid residue R to W and V to A. In the B1 strain, the substitution occurs at the beginning of sgRNA, nucleotide bases AG (on the untransformed calli/controls) to TA causes the stop codon to occur right at the end of sgRNA. From this result it can be ascertained that the B1 strain received the most significant impact of the knock-out system. The MT protein was definitely not working properly on the B1 strain.
Figure 5. Alignment of sgRNA area from oil palm calli transformed by CRISPR-IFR2 module compared with the untransformed callus. (A) DNA sequences. (B) DNA and amino acid sequences. NT: untransformed callus. A1-6: transformed calli from 6 different lines.

Figure 6. Alignment of sgRNA area from oil palm calli transformed by CRISPR-IFR2 module compared with the untransformed callus. (A) DNA sequences. (B) DNA and amino acid sequences. NT: untransformed callus. A1-6: transformed calli from 6 different lines.
4. Conclusions

CRISPR/Cas9 constructs carrying sgRNA with targeted MT and IFR genes were successfully introduced into oil palm callus caused mutations in both targeted DNA regions resulting in alterations on sequences of the amino acid residues. On the selection medium, the transformed calli developed into somatic embryos.

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