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Genome Editing Targeting Flavanone 3-Hydroxylase Knock Out Gene Using CRISPR/CAS9 (pRGEB32-F3H) in Rice Plant Transformation

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Abstract: Sakuranetin is a flavonoid compound predominantly produced by rice plants in response to biotic and abiotic stress. This compound can be induced through stress triggers such as UV radiation, jasmonic acid accumulation, and pathogen infection. Its biosynthesis originates from the precursor naringenin, catalyzed by the OsNOMT gene. However, naringenin can also be converted into dihydrokaempferol by the Flavanone 3-Hydroxylase (F3H) enzyme, affecting sakuranetin production. The conjugation of naringenin by F3H reduces the accumulation of sakuranetin, a phytoalexin crucial for plant stress resistance. Enhancing sakuranetin levels can be achieved by knocking out or silencing genes like F3H through CRISPR/Cas9 genome editing, a transformative genetic engineering method inducing targeted gene mutations. This research focuses on designing sgRNA for the F3H gene to optimize sakuranetin production in rice. The methodology involves selecting F3H sgRNA targets, constructing target gene mutation plasmids, transforming plasmids into Agrobacterium, and subsequently infecting rice explants. The study yielded an F3H sgRNA sequence with 55% efficiency for insertion into the pRGEB32 plasmid. This plasmid was successfully transformed into Agrobacterium, verified by rifampicin and kanamycin antibiotic selection and PCR confirmation. Infected rice explants from the Koshihikari variety displayed positive responses, evidenced by the formation of planlets, signifying the success of the transformation process.

Keywords: Agrobacterium, CRISPR/Cas9, Flavanone 3-Hydroxylase, Genome editing, Rice Plants, Sakuranetin.

INTRODUCTION

The defense response of rice plants when under stress can occur through several mechanisms, one of which is the production of secondary metabolites in the form of flavonoids. Flavonoids are used by rice plants as a defense response to stress conditions, particularly biotic stress from microorganisms such as fungi or abiotic stress such as salinity and drought. One type of flavonoid that is widely produced by rice when attacked by biotic stress from fungi is sakuranetin.

Sakuranetin is a potential metabolite agent whose activation can be triggered by applying stress to plants [1]. Sakuranetin has many health benefits, such as antioxidant, anti-cancer, antiviral, anti-inflammatory, antidiabetic, antimutagenic, and antimicrobial properties [2]. The main precursor of sakuranetin is the compound naringenin, which is catalyzed by the OsNOMT gene [3]. Naringenin, as a flavonoid precursor, is not only used to form sakuranetin but also other flavonoid compounds such as prunin, apigenin, anthocyanin,

and others. The biosynthesis of sakuranetin is influenced by the involvement of the F3H (Flavanone 3-Hydroxylase) gene, which is capable of conjugating the sakuranetin precursor to the dihydrokaempferol and eriodictyol forms.

Flavanone 3-Hydroxylase (F3H) is an enzyme that converts naringenin, a precursor of sakuranetin, into flavonoid compounds. This condition can reduce the optimization of plants to produce sakuranetin as a defense agent for rice plants when they are under stress. Based on several gene regulations, there is a method that can be used to inactivate the F3H gene, namely through gene silencing using genome editing. Genome editing using the CRISPR/Cas9 technique has become an efficient method for modifying target genes, or more specifically, cutting DNA at specific locations. CRISPR/Cas9 technology is capable of targeting specific genes that need to be deleted without affecting other genes [4].

Considering the background mentioned above, this study aims to determine the effect of F3H gene knock-out on sacuranetin biosynthesis.

The findings of this study are expected to provide a scientific basis for the development of genome editing techniques to improve the quality of rice plants.

MATERIALS AND METHODS

The experiment was in Desember 2024 at the Tissue Culture Laboratory and Agrotechnology Laboratory, Department of Agrotechnology, University of Jember, East Java, Indonesia.

Plasmid Construction Targeted sgRNA Selection

The Flavanone 3-Hydroxylase gene sequence was collected from GenBank on the NCBI website and aligned with its conserved sequence in Clustal Omega. The F3H chromosomal DNA sequence was determined by aligning the chromosomal sequence with the mRNA sequence obtained from GenBank. The designed sgRNA must consist of 20 nucleotides (nt) based on the target sequence and contain a Protospacer Adjacent Motif (PAM). The sgRNA was designed using the CRISPR-based website CHOPCHOP V.3, which generates output related to PAM, sgRNA efficiency, GC content, genomic location, number of mismatches, and its location within the coding sequence (CDS). The selected sgRNA sequence was then extended with GGCA at the 5' end and CAAA at the 3' end, resulting in a 23 nt single-stranded sequence at PT. Ilmu Genetika (Indonesia), namely sgRNA-Exon2.The Flavanone 3-Hydroxylase gene sequence was collected from GenBank on the NCBI website and aligned with its conserved sequence in Clustal Omega.

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Transformation of Plasmid pRGEB32-F3H into Agrobacterium

The successfully constructed plasmid was then ligated and transformed into Agrobacterium tumefaciens LBA4404 using the electroporation method. Selection was then carried out by growing the Agrobacterium transformants in LB agar medium with rifampicin and kanamycin antibiotics. Confirmation of the Agrobacterium transformants was performed by PCR analysis using the hygromycin phosphotransferase II (hptII) marker [5].

Table 1: hptII Primer for Plasmid Confirmation

Gene	Primer	Amplicon size (bp)
hptII	Forward 5' TCGGACGATTGCGTCGCATC 3' Reverse 5' AGGCTATGGATGCGATCGCTG 3'	545

Agrobacterium Transformation in Rice Callus

The rice varieties used in the transformation were Ketan Hitam and Koshihikari. Rice callus was first prepared by inducing it on N6D medium for 14 days. The formed callus was then transformed by infecting Agrobacterium LBA4404 by soaking it in a suspension solution with an OD of 0.5-1. The suspension solution consisted of Agrobacterium pellets grown on liquid MS (Murashige and Skoog) medium and supplemented with 100 mM acetocyringone. The resulting infected callus was then grown on a co-culture medium consisting of N6D+acetocyringone for 48 hours at 28°C in the dark. Next, the callus was washed with sterile ddH2O and transferred to an elimination medium consisting of N6D supplemented with cefotaxime and grown in the light for 7 days. Callus regeneration is carried out by growing selected callus on MS medium with Cefotaxime and incubating for 4-6 weeks at a temperature of 28°C in bright conditions until greenspots appear and develop into plantlets. The developed plantlets are then selected on MS medium with the addition of the antibiotic hygromycin. Plantlets that are able to grow are indicated as putative transformant plants.

Data observations taken in this study included cytochemical analysis of callus using 2% acetocarmine and 0.5% Evans blue staining, callus induction percentage, callus overgrowth percentage, callus browning percentage, and greenspot percentage.

$$callus\ induction\ percentage = \frac{total\ callus}{total\ seeds\ planted} \times 100\%$$

$$callus\ overgrowth\ percentage = \frac{total\ callus\ overgrowth}{total\ callus\ infection/regeneration} \times 100\%$$

$$callus\ browning\ percentage = \frac{total\ callus\ browning}{total\ callus\ infection/regeneration} \times 100\%$$

$$callus\ greenspot\ percentage = \frac{total\ callus\ greenspot}{total\ callus\ regeneration} \times 100\%$$

$$callus\ embriogenic\ percentage = \frac{embriogenic\ area}{total\ callus\ area} \times 100\%$$

RESULT AND DISCUSSION

Design of the pRGEB32-F3H Plasmid Construct

Flavanone 3-Hydroxylase was obtained from GenBank on the NCBI website (www.ncbi.nlm.nih.gov/nucleotide/MW752102.1), then its conserved regions were determined to be introns and exons. The sgRNA nucleotides were designed using the CHOPCHOP website software (www.chopchop.cbu.uib.no/results/1735401055386.2693/) to generate information related to PAM, sgRNA efficiency, GC content, genomic location, number of mismatches, and their location within the coding sequence (CDS). A total of 170 sgRNA sequences were identified as potential knockout targets, and the top 5 sequences were selected as the sgRNA targets to be used.

Table 2: Target sgRNA Sequence in Exon 3

Rank	Sequence	Location	GC	MM	Eff.
15	TTCACCGCTACCTCCCTGATTGG	1244367	55	0	49.00
21	GCACATACTGCAAAGAAGTTCGG	1237262	40	0	42.89
46	GGCAGTCCGAACGTCAGCTCTGG	1237113	65	1	45.77
66	TGCAAGTTCTCAAGGAAGGAGG	1237031	50	2	53.53
118	GGCAGACCACGACACGCTGCCGG	1244857	70	1	44.10

Note : GC = Guanine-cytosin content, MM = Mismatch, Eff. = Efficiency.

The F3H gene is located on chromosome 3, which has the locus name LOC_Os03g03034.1 and a length of 9688 bp. The protein length at LOC_Os03g03034 is 342 aa. The sgRNA location was determined using the CHOPCHOP website, so that sgRNA with a length of 20 nucleotides plus 3 PAM nucleotides could be selected. As shown in Figure 4.2, the black arrow indicates the location of the F3H gene sequence in exon-2 with a length of 23nt at coordinate 1244367.

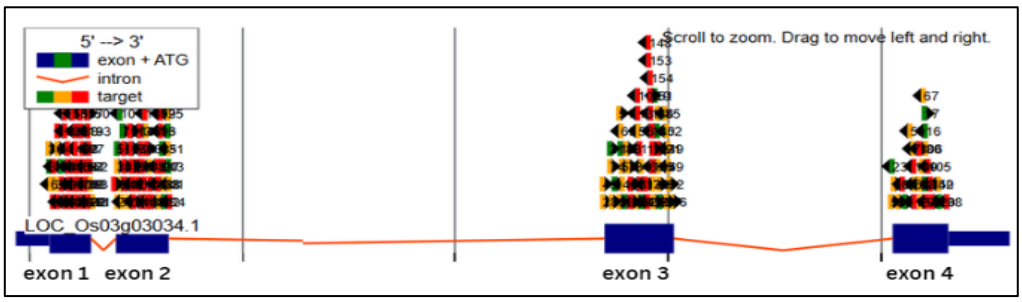

Fig 1: Flavanone 3-Hydroxylase Gene Stucture

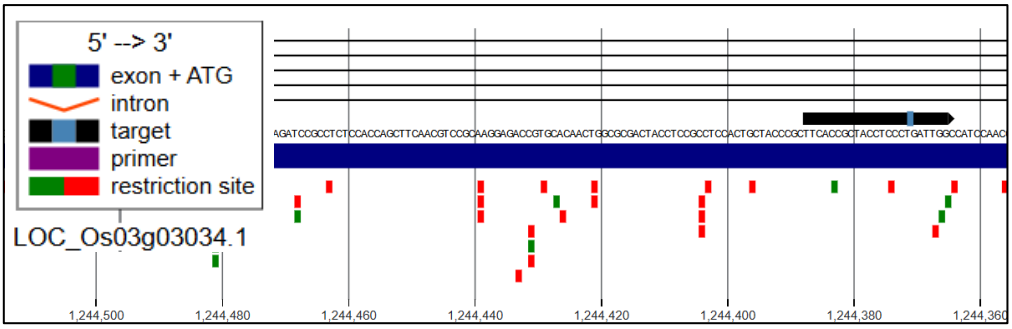

Fig 2: Location of sgRNA F3H on Exon 3

Sequence selection is based on optimal cleavage potential and the resulting knockout effect. The location of cleavage at the beginning (upstream) affects gene silencing efficiency because it causes a faster ORF (Open Reading Frame) shift, thereby halting protein production and gene expression. The efficiency level of the sequence is the ability of sgRNA to guide Cas9 to accurately cut the target DNA; the higher the efficiency, the greater the possibility that Cas9 will be able to cut at the desired location. The efficiency of sgRNA is influenced by the GC percentage. The optimal GC content for sgRNA is around 40-60%, with the highest potential at GC above 50% [6].

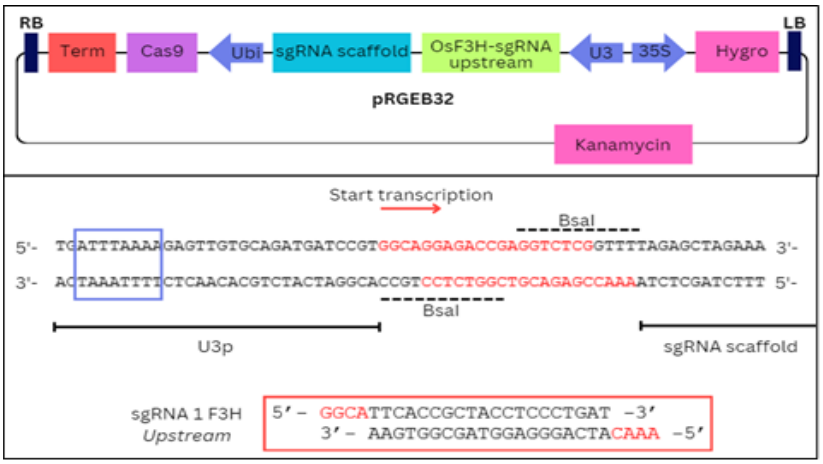

Fig 3: Design Construct Plasmid Knock-Out F3H

Confirmation of Transformant Plasmids using the Polymerase Chain Reaction (PCR) Method

The number of Agrobacterium colonies resulting from transformation was 461 colonies. The Agrobacterium confirmation results showed that it was a transformant, as indicated by the appearance of a DNA band at a length of 545 bp.

Bacterial inoculation in media containing antibiotics showed the growth of 461 single colonies, indicating that plasmid transformation into Agrobacterium using the electroporation method was effective. The characteristics of Agrobacterium single colonies are yellowish-white with a hint of pink, round in shape, and almost transparent [7]. The transformation of Agrobacterium with the pRGEB32-F3H1 plasmid was confirmed using PCR

and electrophoresis, followed by visualization on a UV transilluminator. The primers used in the PCR method were hptII (Hygromycin Phosphotransferase II) primers, which amplified a 545 bp DNA fragment [5].

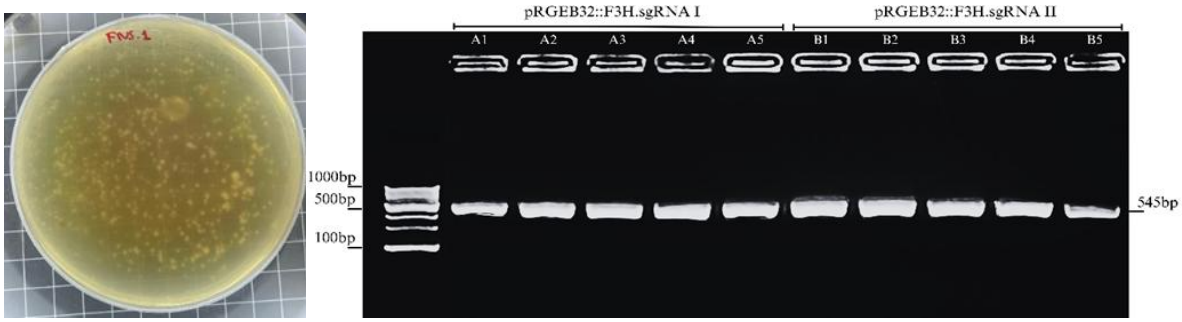

Fig 4: Results of Agrobacterium Tumefaciens LBA4404 Transformation with 461 colonies and PCR confirmation of Agrobacterium transforman

Respon of Callus Transformant

The results of Agrobacterium transformation in callus showed several responses, including: The transformation into rice callus used two rice varieties, namely Koshihikari (Japonica) and Ketan Hitam (Indica). The callus characteristics of the Ketan Hitam variety are yellowish-white with a compact texture, and yellow with a crumbly texture for the Koshihikari variety. These callus characteristics are suitable for transformation because they have embryogenic potential. The variety with the highest callus induction rate is Ketan Hitam, at 97.3%.

Table 3: Percentage of Callus Induction

No.	Variety	Morphology	Number of Explan	Callus Induction
1.	Ketan Hitam	Yellowish-white, compact texture	1200	97.3%
2.	Koshihikari	Yellow, crumbly texture	1200	95.8%

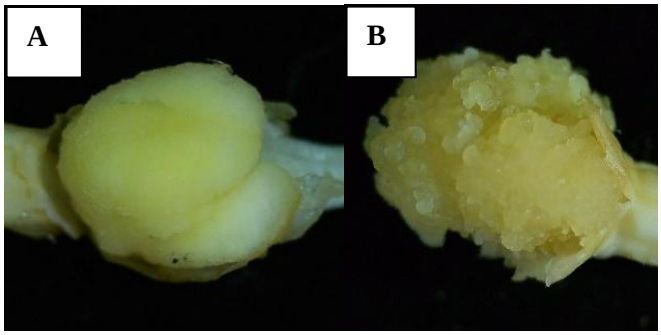

Fig 5: Callus morphology of (A) Ketan Hitam, (B) Koshihikari

This difference is influenced by genetic factors and the concentration of hormones used [8]. Both varieties are compact and yellowish-white in color, indicating good embryogenic properties [9].

Table 4: Percentage of Callus Overgrowth and Browning after Agrobacterium Infection

No.	Variety	Number of Callus	Callus Overgrowth	Callus *Browning*
1.	Ketan Hitam	1168	23.2%	16.8%
2.	Koshihikari	1150	21.5%	12.5%

This overgrowth was caused by excessive Agrobacterium concentration, insufficient rinsing time, and suboptimal drying of the explants. The percentage of browning explants was also highest in the Ketan Hitam variety at 16.8%, while Koshihikari had 12.5% (Table 4). This was caused by high stress accumulation due to Agrobacterium infection.

Table 5: Callus Response During the Regeneration to Selection Stage

No.	Variety	Number of Callus	Callus Embriogrnic	Callus *Browning*	Callus Greenspot	Number of Planlet
1.	Ketan Hitam	700	52.6%	32.4%	-	-
2.	Koshihikari	758	63.1%	23.9%	15.9%	1

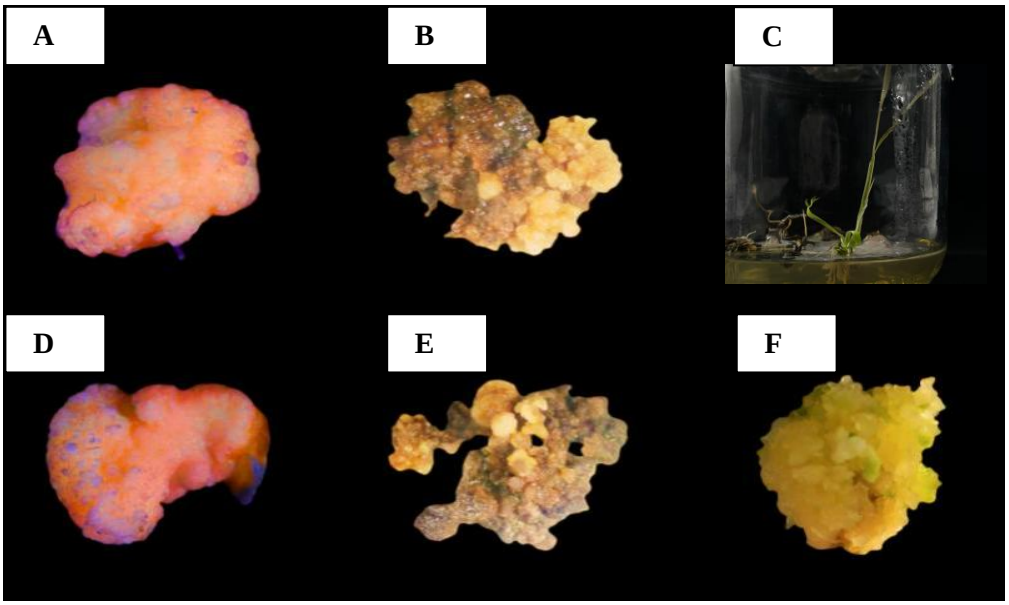

Fig 5: Callus Regeneration – Selection Phase; A: Callus Embriogenic of Ketan Hitam, B: Callus Browning of Ketan Hitam, C : Planlet of Koshihikari, D: Callus Embriogenic of Koshihikari, E: Callus Browning of Koshihikari, and F : Callus Greenspot of Koshihikari.

The embryogenicity of explants can be determined using cytochemical visualization techniques on callus tissue with a 0.5% Evans Blue solution and a 2% Acetocarmine solution. Explants that show a dominant red color after staining are considered embryogenic. Callus with compact characteristics is reactive to Acetocarmine, whereas crumbly callus structure is permeable to Evans Blue solution [10]. Explants that show a positive response during the transformation process are Koshihikari varieties, which are capable of producing 1 planlet that grows well.

CONCLUSION

The design of sgRNA 1 F3H and the construction of the pRGEB32 plasmid with the CRISPR/Cas9 system used to silence the Flavanone 3-HyThe response of transformed rice callus evaluated showed a positive response to Agrobacterium transformation, with Koshihikari variety explants exhibiting a higher percentage of embryogenesis and a lower percentage of browning than the Ketan Hitam variety.droxylase gene were obtained by arranging the target sgRNA at sequence location 230. Further research is needed to optimize the use of varieties and the process of Agrobacterium transformation into callus to obtain more optimal putative transformants.

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