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ENGINEERING OF sgRNA TARGETING DREB4 ISOLATED FROM *ORYZA SATIVA* USING pRGEB32 IN CRISPR/Cas9 GENE EDITING

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SUMMARY

Rice (*Oryza sativa* L.) production on acidic drylands needs optimization through breeding to develop drought-resistant cultivars. The rice cultivar Inpago-5 showed moderate tolerance to drought stress. However, further enhancing this tolerance can proceed by precise gene editing of the DREB (dehydration-responsive element binding) gene, utilizing the CRISPR (clustered regularly interspaced palindromic repeats) /Cas9 system. The presented study aimed to engineer the single-guided RNA (sgRNA) targeting the DREB gene in rice cultivar Inpago-5 using the CRISPR/Cas9 approach. The use of the pRGEB32 plasmid harboring the Cas9 gene helped facilitate the cloning of sgRNA-DREB through the Golden Gate cloning technique and heat shock transformation. The sgRNA-DREB confirmation succeeded through colony PCR (polymerase chain reaction), followed by plasmid isolation and subsequent validation using PCR with specific primers and sequencing primers targeting the pRGEB32 vector. The latest analysis revealed the pRGEB32-sgDREB_sg1 and pRGEB32-sgDREB_sg2 transformed bacteria proliferated on LB+Kanamycin selection media, indicating successful insertion of

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sgRNA-DREB into the pRGEB32 plasmid. The verification through colony and plasmid PCR with specific primers confirmed the presence of a 425-bp amplicon. Sequencing data showed the correct insertion of sgRNA-DREB sequences at the designated site.

Keywords: Rice (*O. sativa* L.), cultivar Inpago-5, plant breeding, CRISPR/Cas9, gene editing

Key findings: The gene encoding rice (*O. sativa* L.) drought tolerance DREB4 succeeded integration into the pRGEB32 vector, with the sgRNA-DREB accurately inserted at the targeted site.

INTRODUCTION

Recent past studies revealed local Indonesian rice (*Oryza sativa* L.) cultivars, particularly the upland cultivars like Inpago-5, exhibited the suboptimal drought tolerance despite high productivity under ideal soil conditions (Fendiyanto *et al*., 2019, 2024). Under suboptimal and arid conditions, the upland rice cv. Inpago-5 productivity displayed a significant decrease compared with its performance under optimal soil conditions. Under drought stress conditions, reports on cultivar Inpago-5 productivity losses stated as much as two tons per hectare. The drought tolerance profile of cv. Inpago-5 on dry and acidic soils was moderate compared with other rice cultivars, such as Hawara Bunar, IR64, Inpago-4, Inpago-7, Inpago-8, and Limboto (Fendiyanto *et al*., 2019; Miftahudin *et al.,* 2021). Consequently, enhancing the drought tolerance of the cultivar Inpago-5 through genetic engineering of drought-responsive genes presents a viable approach.

Genes involved in plant resilience to abiotic stress conditions encompass a diverse array, including *SOD, OsGERLP, OsDREB1, OsDREB2, OsDREB4*, and *OsFRDL4* (Fendiyanto *et al*., 2019, 2024; Miftahudin *et al*., 2021; Satrio *et al*., 2019, 2021, 2023). Among these, the *OsDREB* gene encodes a transcription factor pivotal for drought response in rice. The said gene has emerged to bolster plant resilience against drought and other abiotic stress factors (Tian *et al*., 2005). The *OsDREB* functions as a crucial transcriptional regulator under drought conditions, facilitating the upregulation of downstream genes, such as *PLS1* and *PLS2*. The *OsDREB* exhibited substantial homology with *TaDREB* in wheat, *DREB* in barley and rye, and *zmDREB* in maize. Furthermore, the *DREB* gene in rice was evident as a significant hotspot within quantitative trait loci (QTL) regions governing the drought stress mechanisms (Tian *et al*., 2005).

In the *OsDREB* gene, the genetic modification can progress through contemporary gene-editing technologies, such as, CRISPR/Cas9. However, a paucity of research exists involving the application of CRISPR technology to manipulate the *DREB* gene in rice cultivar Inpago 5. Most studies on CRISPR technology in rice cultivar Inpago-5 and other crops have focused only on in silico analyses (Halim *et al*., 2021; Pratami *et al*., 2022; Fendiyanto *et al*., 2023). To date, the efforts to enhance plant drought tolerance have largely centered on conventional breeding methods, such as cross-breeding, which have proven to be ineffectual (Tanaka *et al*., 2023). Consequently, the presented study aimed to engineer a vector carrying sgRNA-DREB for use in rice cultivar Inpago-5, utilizing the CRISPR/Cas9 system in *Escherichia coli* bacteria. This research represents a critical initial step in the genetic engineering of the drought-resistant rice cultivar Inpago-5.

MATERIALS AND METHODS

Plant and vector materials

The plasmid pRGEB32 served as the vector for the construction of the CRISPR/Cas9 cassette. The sgRNA targeting the *DREB* gene underwent synthesis in rice (*O. sativa* L.) cultivar Inpago-5 with two distinct variants, each comprising 23 base pairs. In the promising study, the additional material utilized included the liquid Luria-Bertani (LB) medium (Sezonov *et al*.,

2007), the Luria-Bertani agar (LA) medium, and antibiotics, such as, kanamycin and streptomycin. Reagents comprised annealing oligonucleotides from NEB (USA) and Golden Gate cloning reagents, also sourced from NEB, USA. Furthermore, utilizing a 0.1 M $CaCl_2$ solution combined with 15% glycerol, nuclease-free water (NFW), and 70% ethanol also occurred. For polymerase chain reaction (PCR) amplification, the use of MyTaq™ HS Red Mix (Bioline, USA), along with agarose and TAE buffer for gel electrophoresis, ensued. The GeneJET Plasmid Miniprep Kit (Thermo Scientific, USA) and the M13 Reverse Primer (5'-CAGGAAACAGCTATGAC-3') were also integral in the said research. The bacterial strains used included *Escherichia coli* strain TOP10 and *Escherichia coli* harboring the pRGEB32 plasmid.

CRISPR/Cas9 vector construction and sgRNA-DREB generation

The engineering of CRISPR/Cas9 editing vectors using sgRNA oligonucleotides involved several critical steps, including phosphorylation, annealing, digestion, and vector ligation. These procedures adhered to the methodology outlined by Kusumawati *et al*. (2023). Initially, 1 µl of sgRNA oligo pairs (100 µM), combined with 2 µl of T4 ligase buffer, 1 µl of T4 polynucleotide kinase (PNK) enzyme, and 15 µl of nuclease-free water (NFW), facilitated the phosphorylation. The reaction mixture then sustained incubation at 37 °C for one hour to activate the enzyme, followed by inactivation at 95 °C for three minutes and cooling to 25 °C. The resultant double-stranded oligonucleotides served for ligation with the pRGEB32 plasmid. In this phase, 1 µl of oligo duplex's incorporation into the Golden Gate master mix reaction comprised 1 µl of pRGEB32 plasmid (75 ng/µl), 1 µl of T4 ligase buffer, 1.25 µl of T4 ligase enzyme, 0.5 µl of BsaI-HF®v2, and 5.25 µl of nuclease-free water (NFW). Conducting the Golden Gate assembly used a Select Cycler-II PCR machine (Select BioProducts, USA), with 25 cycles alternating between restriction at 37 °C for two minutes and ligation at 16 °C for three minutes. Consequently, the integration of the CRISPR/Cas9 vector into the pRGEB32 plasmid ran through a meticulously structured protocol (Kusumawati *et al.,* 2023), ensuring both efficiency and precision in the process.

Introduction of recombinant vectors into *E. coli* TOP10

The transformation process commenced with the preparation of competent *E. coli* TOP10 cells, following the protocol as outlined by Chan *et al*. (2013), with slight modifications by Kang *et al*. (2013). Initiating, the procedure comprised 200 µl of *E. coli* TOP10 overnight culture inoculated into 2 ml of C-Medium combined with 3 ml of the LB medium. Then, the culture's incubation continued in a shaking incubator at 37 °C for 3.5 hours. Subsequently, 1.5 ml of the *E. coli* TOP10 culture reached centrifugation at 4 °C and 4000 rpm for six minutes. The resultant cell pellet's careful resuspension in 100 µl of 0.1 M $CaCl_2$ solution proceeded, then incubated on ice for five minutes. Following re-centrifugation, the cell pellet's meticulous resuspension continued in 100 µl of $CaCl_2$ solution supplemented with 15% glycerol and subsequently preserved at -20 °C (Panja *et al*., 2006; Li *et al*., 2010). The insertion of the recombinant pRGEB32 plasmid into *E. coli* TOP10 took place via the heat shock method, adhering to established protocols (Sambrook *et al*., 1989; Patigu *et al*., 2021). A 10 µl aliquot of the Golden Gate reaction mixture, as combined with 50 µl of competent cells (Zhou *et al*., 2018), attained incubation on ice for 30 minutes. Afterward, a heat shock treatment at 42 °C for 45 seconds followed, with the cells immediately returned to ice for a 10-minute incubation. Subsequently, the introduction of 800 µl of LB medium continued with the mixture shaken at 37 °C for 1.5 hours. The transformation culture's spreading ensued on LB agar plates containing 50 ppm kanamycin, and incubation at room temperature took 48 hours. In conclusion, the transformation process of *E. coli* TOP10, starting from the induction of competency to the insertion of the pRGEB32 plasmid, transpired through a series of steps, ensuring the efficacy and reliability of the transformation.

Selection of positive recombinant colonies in *E. coli*

Screening of colonies of *E. coli* potentially harboring recombinant plasmids employed the colony PCR technique. Two colonies' sampling from each plate used a pipette tip and inoculated into selective media containing 50 ppm kanamycin. The pipette tip, then resuspended in a PCR tube, contained 10 µl of nuclease-free water (NFW). This colony suspension subsequently received 12.5 µl of MyTaq™ HS Red Mix PCR master mix (Bioline, USA), 1.25 µl of specific primers for the pRGEB32 vector (10 µM), and 1.25 µl of the reverse oligo corresponding to the sgRNA insert (10 µM). The procedure commenced with cell lysis at 96 °C for five minutes, followed by 35 amplification cycles, consisting of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. A final post-extension phase at 72 °C for five minutes proceeded. The PCR products then sustained electrophoresis using a 1% agarose gel in 1x TAE buffer at 100 volts for 30 minutes. Visualization of the DNA bands continued using a UV transilluminator to confirm the presence of recombinant constructs.

Plasmid isolation and insertion verification

Transformed *E. coli* TOP10 cells' inoculation into 20 ml of the LB medium supplemented with 50 ppm kanamycin progressed in a 100-ml Erlenmeyer flask. The culture, incubated overnight at ambient temperature under continuous agitation at 200 rpm, used a shaker incubator. Plasmid isolation commenced with the GeneJET Plasmid Miniprep Kit (Thermo Sci, USA) following the manufacturer's protocol. Plasmid concentration quantification continued by calculating the absorbance ratio at 260 nm and 280 nm using a Nanodrop spectrophotometer (Blue-Ray Biotech, Taiwan). Successful integration of the sgRNA sequence into the pRGEB32 plasmid gained verification via the plasmid PCR, employing the M13 reverse primer (5'- CAGGAAACAGCTATGAC -3') alongside the reverse sgRNA oligos for each construct, namely, DREB_F1 (5'-TAGGGGAAGCCGCCTGCCCTGACA -3') and DREB_R1 (5'-AAACTGTCAGGGCAGGCGGCTTCC-3').

The PCR protocol commenced with an initial denaturation at 95 °C for one minute, followed by 35 amplification cycles, each comprising denaturation at 95 °C for 15 seconds, annealing at 60 °C for 30 seconds, and extension at 72 °C for 30 seconds. The process concluded with a final extension at 72 °C for five minutes. The PCR products' subsequent visualization engaged electrophoresis on a 1% agarose gel in 1x TAE buffer, applying a voltage of 100 volts for 30 minutes. The presence of the DREB sgRNA in the positive recombinant plasmid was positive through sequencing at the sgRNA insertion site using the universal primer M13R-pUC (-26), conducted at PT Genetika Science, Jakarta. The resulting sequencing data, encompassing chromatograms and nucleotide sequences, reached sampling to validate the successful incorporation of the sgRNA into the pRGEB32 plasmid.

Data analysis

The verification of sgRNA integration into the pRGEB32 plasmid gained meticulous confirmation through colony PCR, plasmid PCR, and sequencing methodologies. Electrophoresis on 1% agarose gel helped analyze the results of both colony and plasmid PCR, with the outcomes visualized as electrophoregram images. For plasmid sequence analysis, applying the UGENE software version 34.0 continued, with the findings presented in graphical form (Okonechnikov *et al*., 2012). This rigorous and systematic process ensured the successful incorporation of the sgRNA into the pRGEB32 plasmid. All the analyses engaged the R software package (Wickham and Grolemund, 2017; Chang *et al*., 2021), further validating the robustness of the data.

RESULTS

sgDREB and transformation of *E. coli*

The synthesis of sgRNA-DREB in recombinant *Escherichia coli* harboring the pRGEB32 (CRISPR/Cas9 vector) was successful, as carried out in this investigation. The same reached validity by the PCR for sgRNA-DREB, which yielded *E. coli* colonies on Luria-Bertani agar (LA) selection media supplemented with 50 ppm kanamycin and streptomycin (Figure 1A). In this experiment, the positive control was the concentrated pRGEB32-cassette, devoid of sgRNA-DREB. The *E. coli* harboring the pRGEB32-cassette was able to proliferate on the selective medium, as it possesses the kanamycin resistance gene, thereby conferring antibiotic tolerance.

In the presented study, by using *E. coli* strain TOP10, the bacterial strain also exhibited resistance to streptomycin, inherently carries kanamycin resistance. The negative control employed comprised *E. coli* that had not been transformed with either recombinant vectors, thus, failing to survive when cultured on LA media supplemented with kanamycin and streptomycin (Figure 1). The sgRNA-DREB construct map revealed its proximity to the M13 reverse priming site and the *Cas9* coding sequence, along with the kanamycin resistance gene fixed within the pRGEB32 cassette (Figure 1B).

Transformation and PCR of positive colonies

With colony PCR, the transformation efficacy confirmation revealed the presence of colonies harboring the recombinant vector with the DREB sgRNA insert. In the analyzed colonies, 10 (designated as sg1–sg10) displayed apparent insert bands, while the sg11 colony lacked the insertion (Figure 2). The colony PCR

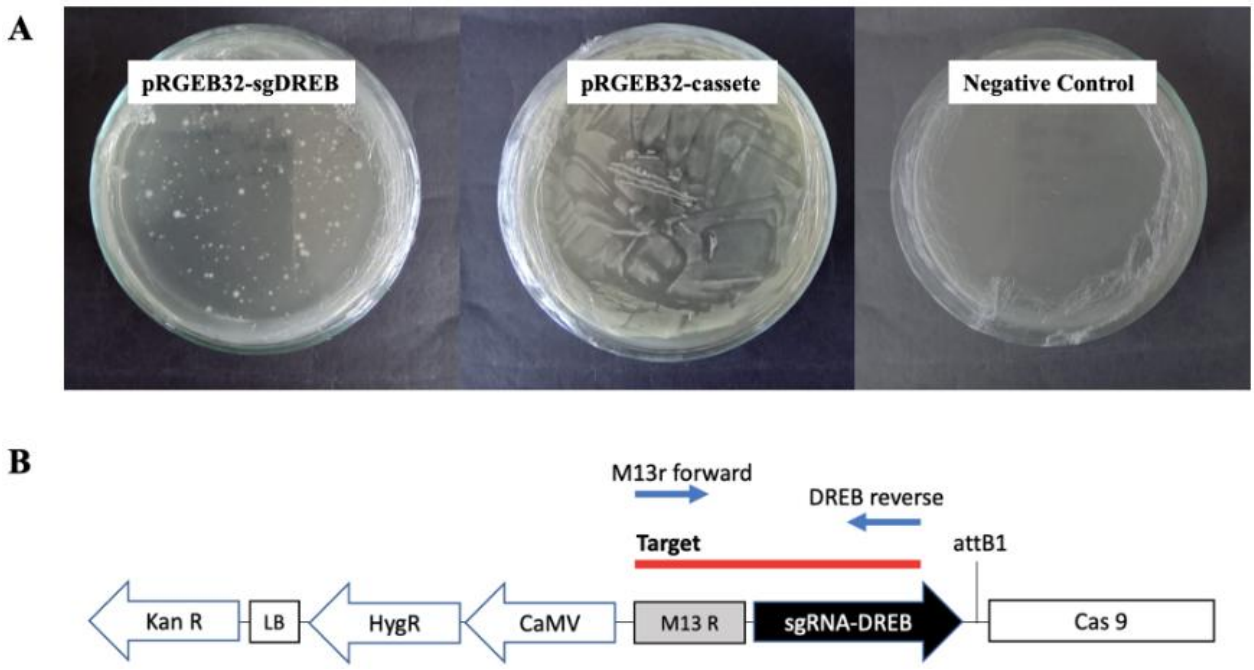

Figure 1. Transformation and construction of sgRNA-DREB within recombinant *Escherichia coli* harboring the pRGEB32 (CRISPR/Cas9 Vector). (A) PCR amplification of sgRNA-DREB colonies; (B) schematic representation of the sgRNA-DREB construct in the pRGEB32 vector. Abbreviations: Kan R-kanamycin antibiotic resistance gene; LB-left border; Hyg R-hygromycin antibiotic resistance gene; CaMV-strong plant promoter; M13R-M13 marker segment; attB1-clonase homologous site; Cas9-a gene encoding the Cas9 protein.

results' visualization used 1% agarose gel electrophoresis, with two negative controls—ddH_2O (to verify the absence of contamination in the PCR reagents) and non-recombinant *E. coli*. The colonies selected for plasmid isolation were sg1 and sg2, both of which were successfully cultured on LB media supplemented with kanamycin and streptomycin. Plasmid PCR subsequently confirmed the presence of the sgRNA insertion in the recombinant pRGEB32 plasmids isolated from the sg1 and sg2 colonies, as also confirmed by the appearance of divergent positive bands (Figure 2).

Validation of sgRNA-DREB insertion sequence

The successful insertion of sgRNA-DREB into the pRGEB32 vector's further substantiation through sequencing analysis authenticated the presence of the sgRNA-DREB insertion site within the recombinant. The sequencing analysis also demonstrated that the pRGEB32 vector was congruent with the designed sgRNA sequence (Figure 3). Moreover, the expected CCTGT cleavage motif was clearly evident within the recombinant pRGEB32 vector, further claiming the accuracy of the insertion (Figure 3B).

DISCUSSION

Dehydration-responsive element binding (*DREB*) genes encode a cohort of transcription factors pivotal in regulating plant responses to environmental stresses, including drought, salinity, and extreme thermal conditions (Yamaguchi and Shinozaki, 1994). In past studies, the DREB, as categorized within the Apetala2/Ethylene Responsive Factor (AP2/ERF) family (Liu *et al*., 1998), exhibited affinity for a specific DNA motif known as the Dehydration-Responsive Element/C-repeat (DRE/CRT) (Sakuma *et al*., 2002) and the situated upstream of genes linked in stress responses (Agarwal *et al*., 2006). These genes'

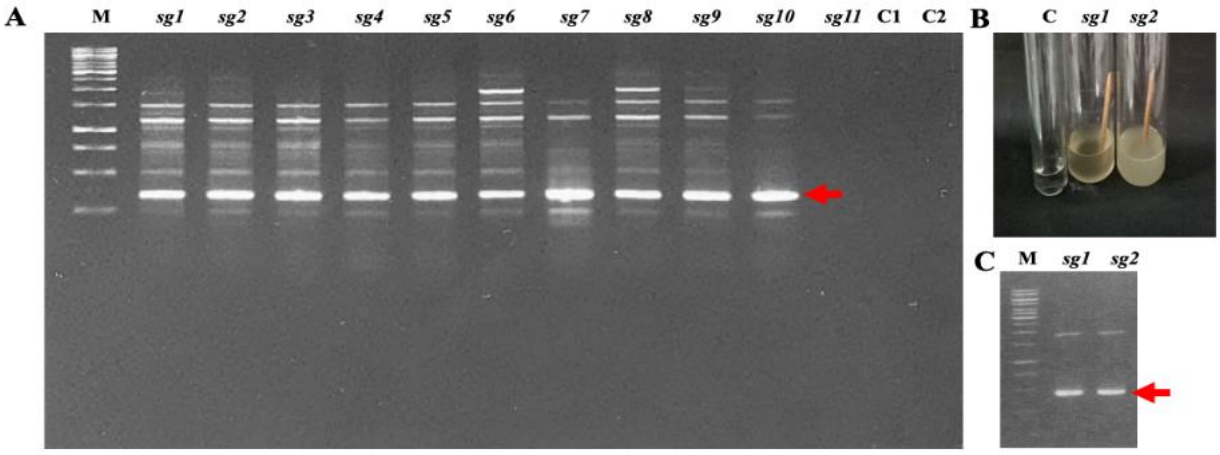

Figure 2. Verification of sgRNA-DREB insertion employing colony PCR and plasmid PCR utilizing M13R-forward and DREB reverse primers: (A) Colony PCR analysis of 11 recombinant *E. coli* colonies, depicting the sgRNA-DREB region. M: 1 kb DNA ladder; sg1-sg11: individual colonies of recombinant *E. coli*; C1: negative control using non-recombinant *E. coli*; C2: negative control with ddH_2O. (B) Selected recombinant *E. coli* cultures harboring the recombinant pRGEB32 plasmid grown on Luria-Bertani Broth (LB) media supplemented with 50 ppm kanamycin. C: control with non-recombinant *E. coli*; sg1: positive recombinant colony 1; sg2: positive recombinant colony 2. (C) PCR analysis of sgRNA-DREB from recombinant pRGEB32 plasmid. M: 1 kb DNA ladder; sg1: recombinant plasmid 1; sg2: recombinant plasmid 2. Red arrows denote positive amplicons corresponding to the M13R region and sgRNA-DREB.

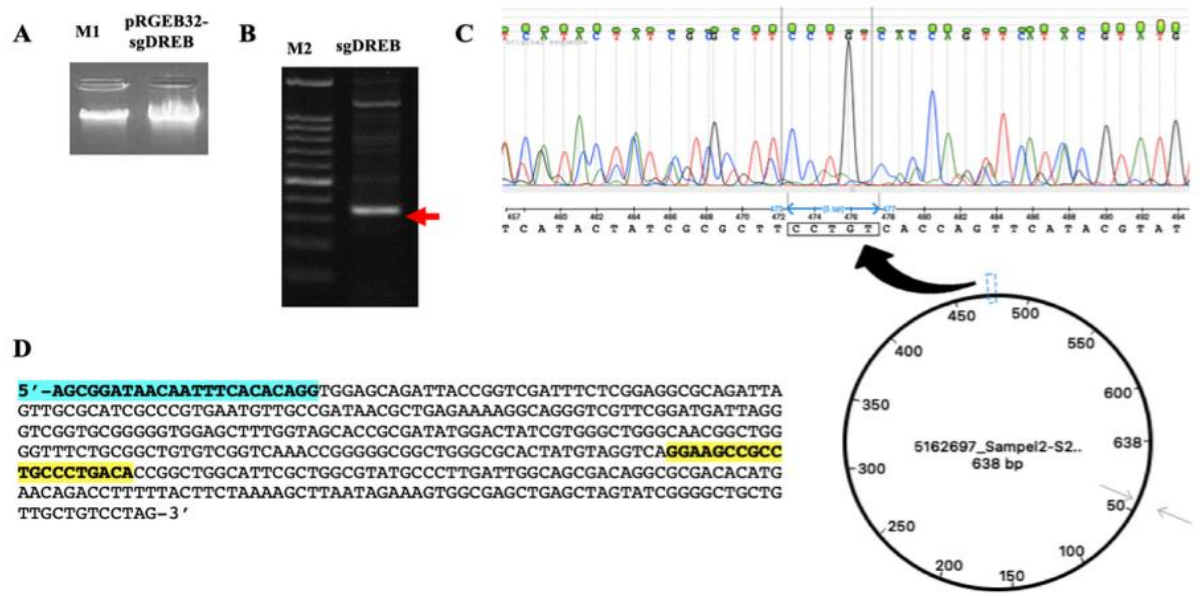

Figure 3. Verification of pRGEB32 insertion through sequencing: (A) Isolation of the recombinant plasmid pRGEB32-sgDREB; (B) PCR verification of the plasmid in the sgDREB region; (C) Partial construction map of the recombinant pRGEB32 vector; (D) Sequence analysis of the recombinant plasmid pRGEB32 and the sgDREB insertion region. M1: lambda DNA marker; M2: 100 bp DNA ladder.

activation further facilitates plant resilience under adverse environmental and stress conditions, particularly in rice. Currently, the employed genes function as drought-responsive elements in rice. The construction of the pRGEB32 plasmid vector, aimed at the *DREB* gene, was also an achievement in this study (Figure 1). This vector construction bore validity through the confirmation of recombinant *E. coli*, colony and plasmid PCR, and sequencing of the recombinant vector pRGEB32-sgDREB.

This study further elucidated the successful construction of the CRISPR/Cas9-sgRNA recombinant vector through the insertion of DREB4 sgRNA into the pRGEB32 vector (Figures 1 and 2). The successful integration of DREB sgRNA began with the synthesis of a sgRNA duplex oligonucleotide. In the presented process, the two complementary oligonucleotides hybridized to form a duplex, which had subsequent phosphorylation by the polynucleotide kinase (PNK) enzyme at 37 °C. Following phosphorylation, further inactivation of the enzyme was at 95 °C. The addition of a phosphate group at the 5′ terminus using T4 PNK (NEB, USA) sought to enhance the efficiency of the ligation process (Si *et al*., 2023). The ligation process was crucial because the phosphorylated 5′ end of the oligo duplex becomes harmonious with the cohesive ends of the pRGEB32 plasmid, which has been digested with the BsaI enzyme.

Consequently, the nitrogenous bases can pair through hydrogen bonding for forming a stable DNA structure, facilitated by the ligase enzyme. The DREB sgRNA oligo duplex, as incorporated into the pRGEB32 plasmid via the Golden Gate method, facilitated coinciding cleavage and ligation in a single reaction. This cloning strategy gained fame for its efficiency and precision for yielding stable recombinant DNA. In ensuring the stability of the final product, the pivotal factor was the precise positioning of the cleavage site on the vector. It generated complementary sticky ends to facilitate the ligation of the sgRNA fragment in the accurate orientation during the assembly process. Consequently, the restriction enzyme BsaI, characterized by a six-nucleotide recognition site (5′-GGTCTC…-3′), was the option. This enzyme belongs to the type IIS

restriction enzyme group, allowing the ligation of sgRNA without the incorporation of extraneous nucleotide sequences by cleaving outside its recognition site (Valla and Lale, 2014). The BsaI cleavage site within the pRGEB32 vector remained at nucleotide positions 9738-9743 on the 3' strand and 9746-9751 on the 5' strand, thereby positioning the sgRNA attachment at nucleotide 9737, with the same also illustrated in the physical map of the pRGEB32_DREB.

The elimination of the type IIS recognition site following the sgRNA ligation precluded subsequent cleavage by the restriction enzyme, thereby preserving the stability of the resultant ligation product. The DNA ligase enzyme facilitated the covalent joining of the termini of single-stranded DNA within double-stranded DNA (dsDNA) molecules. The ligation process requires the presence of a phosphate group at the 5' end and a hydroxyl group at the 3' end. Notably, the DNA ligase exhibited better capability in forming phosphodiester bonds at sticky ends than blunt ends. All procedural stages—including oligo duplex formation, transformation, colony and plasmid PCRs, and the sequencing—demonstrated the vector's successful construction (Figures 2 and 3). Furthermore, verification of insertions within plasmids in CRISPR vector construction could be proficient not only through conventional PCR but also via real-time PCR (Hou *et al*., 2016).

For plasmid DNA, the plasmid pRGEB32_DREB incurred subsequent sequencing. This executed sequencing employed the Sanger sequencing methodology with the universal primer M13R-pUC (-26) (5'-CAGGAAACAGCTATGAC-3'). The sequencing results of the recombinant plasmid DNA indicated the presence of the DREB sgRNA sequence at the correct locus and in the proper orientation (Figure 3). Facilitating the DREB sgRNA detection used the sequence search tool within the UGENE software. The sequence analysis exhibited exceptional reading quality, characterized by pronounced and distinct graph peaks (Figure 3). The development of the said construct represents a preliminary investigation aimed at engineering drought-tolerant rice, specifically targeting the *DREB* gene family, with particular emphasis on the *DREB4-1* gene.

The *OsDREB4-1* is a gene identified in rice (*O. sativa*) that encodes a protein featuring the AP2 domain, which functions as a transcription factor binding to the dehydration response element (DRE). The said gene is a DREB transcription factor family member that is crucial in mediating the gene expression in response to environmental stressors, such as dehydration and salinity (Tian *et al*., 2005). In a greater analogy with these findings, several other genes also exhibited positive responses to the abiotic stress conditions in various crop plants, such as OsSOD in rice (Fendiyanto *et al*., 2021; Satrio *et al*., 2024) and LCYB in banana (Fendiyanto *et al*., 2023). The *OsDREB4-1* gene specifically binds to the DRE element as a crucial component of the promoter regions of genes implicated in stress responses.

Furthermore, the DREB transcription factors, including OsDREB4-1, are integral to the modulation of stress responses and their expression profiles. Notably, the OsDREB4-1 expression, as induced under dehydration and high salinity conditions, underscored its role in aiding plant adaptation to abiotic stress. In contrast, other *DREB* genes, such as *OsDREB1-1* and *OsDREB4-2*, also exhibited immanent expression and continuously expressed regardless of specific stress conditions (Tian *et al*., 2005). In normally growing rice plants, OsDREB4-1 demonstrated the highest expression levels in roots, stems, and spikes, with diminished expression observed in leaves and undetectable levels in sheaths. Moreover, the *DREB* genes are integral to the stress response pathways (Tian *et al*., 2005). The *OsDREB4-1* gene operates as a transcription factor within the DRE/DREB-regulated stress response pathway, which is crucial for modulating the expression of genes that facilitate plant survival under adverse environmental conditions (Tian *et al*., 2005). Collectively, the *OsDREB4-1* gene serves as a pivotal element in the plant's mechanisms for responding to environmental stress conditions, particularly concerning dehydration and salinity.

CONCLUSIONS

The promising study has successfully integrated DREB-1 sgRNA into the pRGEB32 plasmid in rice (*O. sativa* L.) cultivar Inpago-5. This achievement's realization, through the Golden Gate methodology, utilized a temperature regimen of 37 °C and 16 °C for 25 cycles. The verification of the insertion ensued by employing colony PCR and sequencing techniques, which confirmed the sgRNA sequence insertion at the designated locus. Furthermore, the research effectively introduced the recombinant vector into *E. coli* TOP10 bacterial cells.

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