BIODIVERSITAS
Volume 26, Number 2, February 2025
Pages: 920-927

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d260241

Construction of a CRISPR/Cas9-mediated genome editing system in manipulating *OsART1* from *Oryza sativa* cv. Inpago 5

MIFTAHUL HUDA FENDIYANTO[1], EKO SETIAWAN[2,♥], MENTARI PUTRI PRATAMI[3], IFAN RIZKY KURNIYANTO[4], FANDRI SOFIANA FASTANTI[3]
[1]Department of Biology, Faculty of Military Mathematics and Natural Sciences, Universitas Pertahanan Republik Indonesia. Indonesian Peace and Security Center Complex (IPSC), Sukahati, Bogor 16810, West Java, Indonesia
[2]Natural Resource Management, Faculty of Agriculture, Universitas Trunojoyo Madura. Jl. Raya Telang, Bangkalan 69162, East Java, Indonesia. Tel.: +62-313-011146, ♥email: e_setiawan@trunojoyo.ac.id
[3]Research Center for Biosystematics and Evolution, National Research and Innovation Agency of Indonesia. Jl. Raya Jakarta-Bogor Km. 46, Cibinong, Bogor 16911, West Java, Indonesia
[4]Department of Agribusiness, Faculty of Agriculture, Universitas Trunojoyo Madura. Jl. Raya Telang, Bangkalan 69162, East Java, Indonesia

Manuscript received: 30 September 2024. Revision accepted: 18 February 2025.

Abstract. *Fendiyanto MH, Setiawan E, Pratami MP, Kurniyanto IR, Fastanti FS. 2025. Construction of a CRISPR/Cas9-mediated genome editing system in manipulating OsART1 from* Oryza sativa *cv. Inpago 5. Biodiversitas 26: 920-927.* Understanding the mechanism of aluminum (Al) tolerance in rice (*Oryza sativa*) cv. Inpago 5 can be done by mutating the ART1 gene to see which Al tolerance genes have decreased expression globally. This study aimed to construct a pRGEB32 vector containing sgRNA-ART1 derived from rice cv. Inpago 5. The construction of the pRGEB32-sgART1 vector was carried out by designing sgRNA, restriction-ligation, transformation, plasmid isolation, verification of target inserts, and sequencing of sgRNA-ART1. The results indicate that the construction of the recombinant vector pRGEB32-sgRNAART1 was carried out by combining digestion and ligase reactions. The construction of the recombinant pRGEB32 construction showed a high level of success in this study. The success of the ART1 sgRNA amplicon in recombinant *Escherichia coli* indicated this. Annotation studies showed that the pRGEB32 cassette plasmid sequence showed more than 99% homology with the recombinant pRGEB32-sgART1 sequence, especially only 1% was different, namely 20 bp of sgRNA that appeared different. After checking with alignment on the plasmid annotation using the MAAFT alignment method, it was found that 20 bp of ART1 sgRNA was successfully inserted into the pRGEB32-sgART1 recombinant vector. In conclusion, the construction of pRGEB32-sgART1 in this study was successful and showed the presence of a 425 bp band amplicon for the insertion from the region between M13 reverse and sgRNA-ART1.

Keywords: Aluminum, CRISPR/Cas 9, gene editing, pRGEB32, rice cv. Inpago 5

Abbreviations: Al: Aluminum; sgRNA: Single guide RNA; LA: Luria Bertani Agar; pRGEB32: Vector name

INTRODUCTION

Rice (*Oryza sativa* L.) is a cereal plant that has a high level of tolerance to aluminum (Al) stress (Miftahudin et al. 2002, 2007). The creation of rice plant genotypes that are tolerant to Al stress has been successfully created in various ways recently, such as through conventional plant breeding and genetic engineering (Ma et al. 2014; Fendiyanto et al. 2019a; Jingguang et al. 2020). Rice from the Japonica subspecies is reported to have a high level of tolerance when compared to rice from the Indica subspecies (Kochian 1995; Kochian et al. 2015; Jingguang et al. 2020). Tropical japonica rice is also reported to have a higher level of tolerance when compared to other rice cultivars (Kochian et al. 2004). One example of tropical japonica rice is cv. Hawara Bunar. Other rice cultivars are reported to have moderate levels of tolerance, such as rice cv. Inpago 4, Inpago 6, Inpago 7, and Inpago 10 (Fendiyanto et al. 2019b). On the other hand, rice cv. IR64, which is included in the Indica subspecies of rice, is classified as rice that is sensitive to Al stress (Fendiyanto et al. 2019b, 2021). The three types of rice (Japonica, Tropical Japonica, and Indica), based on their tolerance levels to Al stress, are equally needed for the development of new rice lines that are more resistant to Al stress, especially for plant breeding (Nguyen et al. 2001; Tanaka et al. 2023; Satrio et al. 2024).

Plant breeding with gene editing technology mediated by CRISPR/Cas 9 is a good choice in developing rice lines that are resistant to Al stress and studying the system and mode of action of a gene related to plant tolerance in dealing with Al stress (Si et al. 2023). Several genes reported to play a role in the level of rice tolerance to Al stress, such as *ART1* (Kochian et al. 2015), *ART2* (Che et al. 2018), *OsGERLP* (Miftahudin et al. 2021), *OsFRDL4* (Ma et al. 2014; Li et al. 2018; Jingguang et al. 2020), *STAR1* (Yamaji et al. 2009), *STAR2* (Ma et al. 2014; Jingguang et al. 2020), *B11* (Fendiyanto et al. 2019a; Miftahudin et al. 2021), and *OsCDT* (Kochian et al. 2015).

The *ART*1 protein is reported to interact closely with the *STAR1* and *STAR2* genes and regulates many other tolerance genes (Kochian et al. 2015). The use of the *ART1* gene in the study of rice tolerance to Al stress in rice cv. Inpago 5 needs to be done to determine the differences in the transgene system in these genotypes in dealing with resilience to abiotic stress, especially Al stress; research on *ART1* gene manipulation in Indica rice subspecies, especially rice cv. Inpago 5 has never been done before. In fact, the *ART1* gene is reported to have an important role as a transcription factor for plant tolerance to Al stress in Japonica rice subspecies (Ma et al. 2014). The construction of recombinant *pRGEB* constructs has been previously carried out in rice, namely using the *Tad1, OsDREB,* and *Tn1* genes in Indica rice (Halim et al. 2021).

The *pRGEB32* vector is an expression vector that has a gene that encodes the Cas 9 protein that is able to edit genes and make random mutations precisely in monocotyledonous plants, which is then referred to as Non-Homologous End Joining (NHEJ). Cutting with NHEJ produces various types of mutations that cause changes in the functional region of the gene, resulting in changes in the level of Al tolerance from Al tolerant to Al sensitive (Kochian et al. 2015) in the rice cultivar cv Inpago 5. Therefore, this study aimed to create a *pRGEB32* recombinant vector construct containing *sgRNA-ART1* derived from the genomic DNA of rice cv. Inpago 5.

MATERIALS AND METHODS

Plant materials

The plants used in this study were rice cv. Inpago 5. We got rice cv. Inpago 5 from *Balai Besar Penelitian Padi Muara* (Bogor, Ministry of Agriculture). Cv. Inpago 5 rice is classified as upland rice and Indica subspecies. In this research, Rice cv. Inpago 5 grew in acidic and dry soil to test the tolerance level of aluminum stress. Rice was planted in a greenhouse with acidic soil treatment (Fendiyanto et al. 2024) and using minimum nutrient culture (Fendiyanto et al. 2019a).

Procedures

DNA extraction

Fresh leaves (5 g) of the Inpago 5 genotype rice were extracted for DNA using the CTAB method with slight modifications following the method of Fendiyanto et al. (2019b). The modification was the use of chloroform: isoamyl alcohol (C: I) thrice and precipitation using Isopropanol twice during the DNA extraction processes.

The DNA of cv. Inpago 5 rice was then amplified with the *ART1* gene to determine the homology of the gene with the ART1 gene from Japonica rice. The functional region of the *ART1* gene was studied and used as a reference for designing sgRNA-ART1 following the method of Pratami et al. (2022). We used chopchop software and predict the sgRNA oligoduplex sgRNA-ART1 forward (5’-`TAGG TTC AAG GTA TGG ACT AAC GG`-3') and sgRNA-ART1 reverse (5’-`AAA CCC GTT AGTC CAT ACC TTG AA`-3').

Insertion of sgRNA generation, recombinant vector construction, and insertion confirmation

The production of sgRNA from ART1 (sgRNA-ART1) was carried out following the methods of Halim et al. (2021) and Fendiyanto et al. (2023). The vector used to perform gene editing in this study was pRGEB32 (Addgene, USA). The production of the pRGEB32 recombinant vector was carried out using the principle of ligase and restriction with the golden gate systems technique, where the ligase and digestion reactions were carried out at the same time. The pRGEB32-sgRNA-ART1 vector was then transformed into *Escherichia coli* strain dh5α (Zhou et al. 2018). The recombinant *E. coli* bacteria that successfully grew were then checked to determine whether they contained the recombinant pRGEB32 plasmid or not through the colony PCR technique and 50 ppm kanamycin antibiotic selection.

The recombinant vector was introduced into *E. coli* using the TransformAid Bacterial Transformation Kit (Thermoscientific, USA) according to the manufacturer's protocol with slight modifications following Kang et al. (2013) and Li et al. (2010). The recombinant *E. coli* bacteria were verified using the PCR-colony technique, particularly by adding a PCR reaction mix consisting of 1 μL of M13R forward primer (5'-`CAG GAA ACA GCT ATG AC` -3'), 1 μL of sgRNA-ART1 reverse primer (5'-`AAA CCC GTT AGTC CAT ACC TTG AA`-3'), 5 μL of PCR mix (taq polymerase) (MyTaq HS Red Mix, Meridian Bioscience, USA), and 3 μL of ddH_2O containing a sample of the recombinant bacterial colony. The PCR product was then run with 1% (w/v) agarose (1st BASE, SNG) using 1X Tris-Borate-EDTA (TBE) Buffer (1st BASE, SNG). The electrophoresis results were then visualized with a UV-transilluminator under UV-B and blue light (Thermo Fisher Scientific, USA).

Plasmid isolation and insertion verification

Recombinant bacteria containing the recombinant plasmid pRGEB32-sgRNA-ART1 were then isolated using the GeneJET plasmid miniprep Kit (Thermoscientific, USA). The successfully isolated recombinant plasmid was then amplified by PCR (predenaturation 95°C for 5 mins, denaturation 95°C for 30 sec, annealing 55°C for 15 sec, extension 72°C for 30 sec, and post-extension 72°C for 5 mins), and the cycle was 35x from denaturation to extension stages (Panja et al. 2006; Hou et al. 2016; Kusumawati et al. 2023) using 1 μL of M13R forward primer (5'-`CAG GAA ACA GCT ATG AC` -3'), 1 μL of sgRNA-ART1 reverse primer (5'-`AAA CCC GTT AGTC CAT ACC TTG AA`-3'), and 5 μL of PCR mix (taq polymerase) (MyTaq HS Red Mix, Meridian Bioscience, USA). The total volume of PCR mix used was 10 μL. Verification of the insert orientation was also carried out (Sambrook et al. 1989) by comparing the sgRNA-ART1 forward primer and the M13 primer. Plasmids containing the correct orientation were then sequenced using a DNA sequencing company (1^{st} BASE, Singapore).

Data analysis

Plasmid sequencing was performed in two directions, namely from the positive strand using the M13R-forward primer and from the negative strand using the sgRNA-ART1 reverse primer. The recombinant plasmid sequence obtained was then contig and analyzed for sgRNA insertion using the UGENE program (Uniprot, USA) (Okonechnikov et al. 2012). Alignment with the pRGEB32 vector sequence was performed using the Ugene program with the MAFFT method. We compared the recombinant plasmid sequence with the pRGEB32 backbone plasmid sequence (Addgene, USA).

RESULTS AND DISCUSSION

Construction of pRGEB-art1 recombinant

The construction of the recombinant vector pRGEB32-sgRNAART1 was carried out by combining digestion and ligase reactions. The restriction enzyme used in this study was Bsa1. The construction of sgRNA-ART1 was also made by adding additional nucleotide regions so that the ligase process between sgRNA-ART1 and the pRGEB32 vector had a high affinity. The polynucleotide kinase (PNK) enzyme was also added in this study to increase the affinity ligase process, namely in the process of combining the 5-P and 3-OH ends of both sgRNA and the pRGEB32 vector. The construction of recombinant pRGEB32 showed a high level of success in this study. This was indicated by the successful presence of ART1 sgRNA amplicon in recombinant *Escherichia coli*. Of the total 7 colonies of *E. coli* bacteria that successfully passed the 5 ppm kanamycin antibiotic selection, there were 6 positive colonies containing the ART1 sgRNA insert (Figure 1.A). The control was nonrecombinant pRGEB32 plasmid (C1), pRGEB32-sgDREB as negative control (C2), along with ddH2O (C3) showed no ART1 sgRNA band (Figure 1.A). The recombinant bacteria that successfully grew were then re-selected using Luria-Bertani agar (LA) media supplemented with 50 ppm kanamycin antibiotic. The six positive bacteria successfully grew on LA + 50 ppm Kanamycin selection media (Figure 1.B). In addition, nonrecombinant bacteria containing the pRGEB32 cassette (without sgRNAART1, positive control) also successfully grew on the selection media (Figure 1C). In contrast, nonrecombinant *E. coli* bacteria failed to grow on LB selection media supplemented with 50 ppm kanamycin antibiotic (Figure 1.D).

Plasmid isolation and verification of sgART1 insertion

Isolation of the recombinant plasmid pRGEB32-sgART1 is important to confirm the success of the vector construction process. The recombinant *E. coli* bacteria that were successfully selected were then cultured on Luria Bertani Agar (LA) selection media (Sezonov et al. 2007), which was given a 50 ppm kanamycin antibiotic. The selected bacteria came from colonies 1 and 2. There was a difference in colony growth rate as indicated by the relatively different turbidity of the colonies from both (Figure 2.A). Colony 1 tended to grow faster, so it was selected for plasmid isolation. The results of the recombinant plasmid isolation were successfully carried out using the heat-shock isolation technique, and the plasmid band from the pRGEB32-sgART1 plasmid isolation results showed two clear supercoiled bands, indicating that the recombinant plasmid isolation process was successfully carried out specifically (Figure 2.B). The plasmid obtained was then selected to amplify the insert using a pair of verification primers and, based on the sgART1 reverse and M13r forward primers, showed the correct sgRNA orientation as indicated by the appearance of an amplicon band of 425 bp (Figure 2.C).

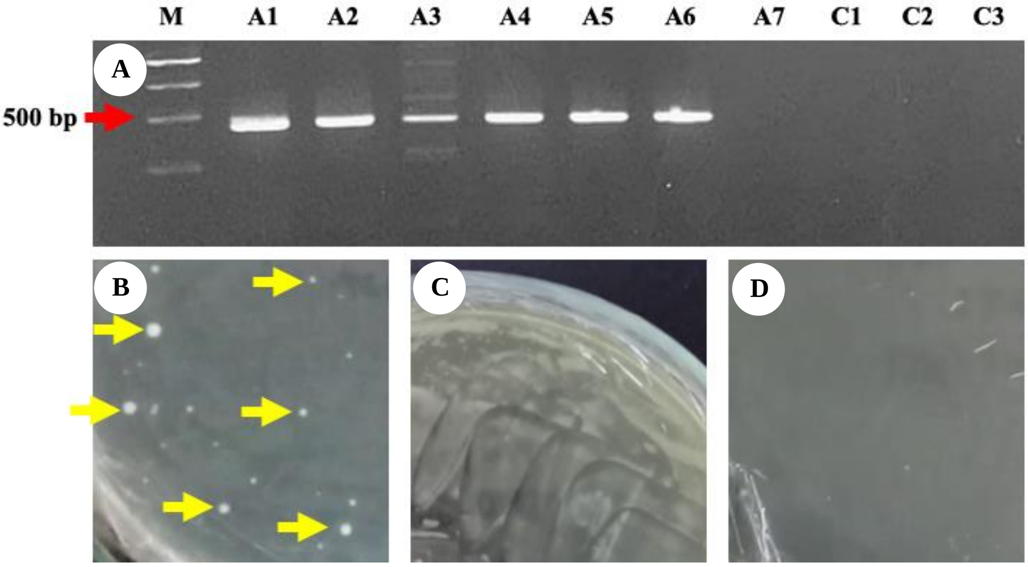

Figure 1. sgRNA generation and transformation in recombinant *Escherichia coli* containing pRGEB32-sgART1: A. PCR of ART1 sgRNA colonies; B. *E. coli* colonies transformed by ART1 sgRNA; C. Positive control *E. coli* containing empty pRGEB32 cassette; D. Negative control *E. coli* without pRGEB32-sgART1. ART1 sgRNA was amplified using sgART1 reverse and M13r forward primers with a size of 425 bp. M: 1 kb marker; A1-A7: Recombinant bacterial colonies; C1-C3: negative control

The recombinant pRGEB32 construction map can be shown with the M13 R and attB1 flanking regions (Figure 2.D). Important sites that can be found in the pRGEB32 vector include Kan R, LB, HygR, CaMV, M13 R, sgRNA-ART1, attB1, and Cas 9. The Kan R site shows a sequence that encodes resistance to the antibiotic kanamycin. The LB site is a left border site, where this area is a specific recombination site for insertion of regions that are homologous to the plant genome so that the sites flanked by the right border (RB) can be inserted into the nuclear genome. The HygR site is a region that encodes plant resistance to the antibiotic hygromycin. The CaMV region is a gene that encodes a strong promoter derived from the Cauliflower Mosaic Virus (CMV) commonly used for plants. The universal M13 region is a general region found in plasmids in general. The sgRNA-ART1 site is a target region inserted into the pRGEB32 vector. The attB1 site is a homologous region used for the introduction of the BP-clonase enzyme to clone a specific gene with a gateway system. The Cas9 sequence is a site that encodes the Cas 9 protein that can perform gene editing on the pRGEB32 vector (Figure 2.D).

Sequencing and analysis of sgART1 target on recombinant plasmid pRGEB32

Bioinformatics study on pRGEB32 plasmid annotation is important to be conducted. Annotation study showed that the pRGEB32 cassette plasmid sequence from AddGene (USA) showed more than 99% homology with the recombinant pRGEB32-sgART1 sequence, especially only 1% difference, namely 20 bp of sgRNA that appeared different (Figure 3A, 3B). Important sites that were successfully mapped from the pRGEB32 sequence from Addgene were T-DNA RB, Cas 9, UBI p, U3p, gRNA, 35S, HPTII, T-DNA LB, and Kan. The size of the recombinant pRGEB32 vector was 15.9 kb (Figure 3.A). The U3p and UBI p sites are important sites that encode the promoter and ubiquitin gene. The sgRNA site of ART1 has also added 5'-CAAA-3' at the 3' end for the Bsa1 restriction recognition site (Figure 3.C). After checking with alignment on plasmid annotation using the MAAFT method, it was found that 20 bp of ART1 sgRNA was successfully inserted into the recombinant vector pRGEB32-sgART1 (Figure 3.D).

Discussion

Rice cv. Inpago 5 is an upland rice that can grow well on suboptimal soil. Based on physiological studies, Inpago 5 rice has a unique tolerance mechanism in responding to aluminum (Al) stress, especially by making the photosynthesis process (Fendiyanto et al. 2024) and photosynthetic pigment synthesis relatively stable when stressed by Al (Fendiyanto et al. 2019a). Cv. Inpago 5 is a cultivated variety that has high productivity and is moderately tolerant when grown on low pH media (3-5 pH) with high Al content. Inpago 5 rice can have relatively high productivity, reaching 8 tons/ha even though it is grown on acidic soil. This ability is related to the tolerance mechanism of the rice when planted on suboptimal soil (Fendiyanto et al. 2019a); the study of cv. Inpago 5 rice, when stressed by Al and its mechanisms, is important to study.

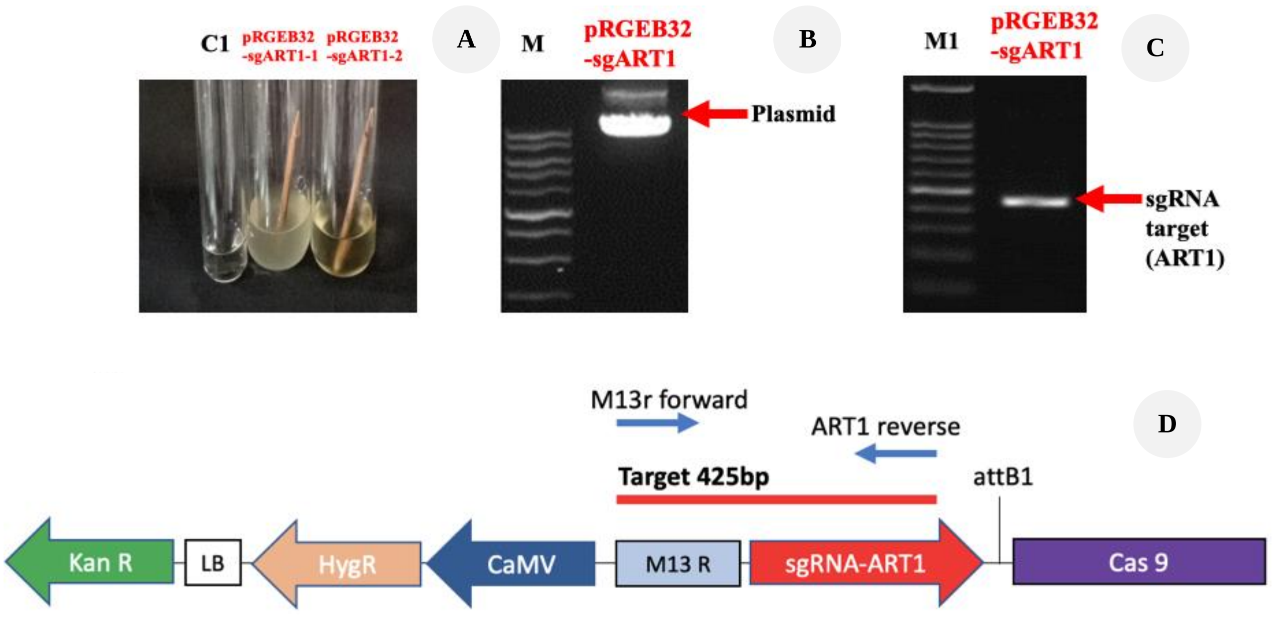

Figure 2. Isolation of recombinant plasmid pRGEB32-sgART1 and verification of sgRNA insert: A. Colony of recombinant *E. coli* pRGEB32-sgART1; B. Visualization of recombinant plasmid pRGEB32 sgART1; C. Target band of sgART1 using recombinant plasmid template; D. Construction map of pRGEB32 vector containing sgRNA-ART1 insert

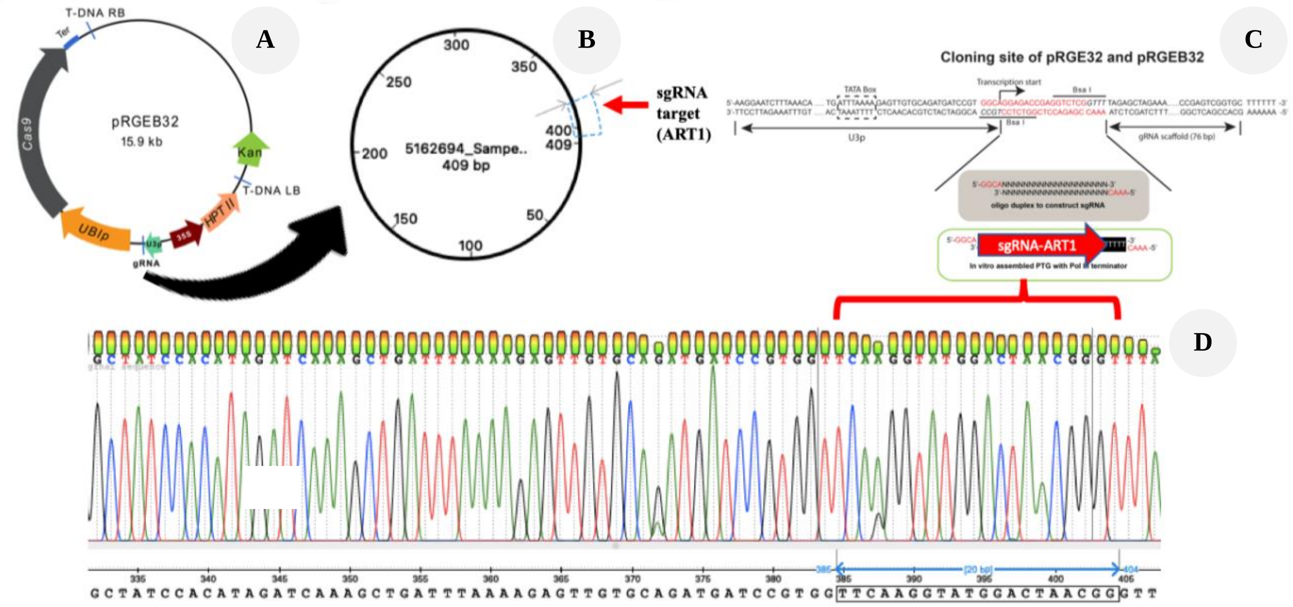

Figure 3. Sequence of sgRNA-ART1 in construct pRGEB32: A. Construction of plasmid pRGEB32 with sgRNA main region; B. Target sgRNA sequence from sequencing scheme; C. sgRNA cloning site region of ART1 in recombinant pRGEB32 vector; D. Position of sgRNA-ART1 sequence with chromatogram quality

The mechanism of rice tolerance to abiotic stress is complex (Liu et al. 1998; Sakuma et al. 2002; Tian et al. 2005; Satrio et al. 2019, 2021, 2023, 2024). Al tolerance in rice is still investigated using molecular and physiological approaches (Meng et al. 2017; Awasthi et al. 2019; Saha et al. 2019; Jingguang et al. 2020); the process of revealing the mechanism of rice tolerance cv. Inpago to Al stress can be done by having a tolerant cultivar Inpago 5, for example, by overexpressing the Al tolerant gene (Zhang et al. 2016), and a sensitive cultivar Inpago 5. Studies on the creation of Al tolerant rice in Indica and japonica rice have been widely carried out (Miftahudin et al. 2021). However, studies on the creation of Al-sensitive mutants have been very few. This study conducted the creation of ART1 knock-down mutants to study the relationship between the *ART1* gene and other tolerance genes (Fendiyanto et al. 2019b; Miftahudin et al. 2021) and to find other candidate responsive genes in the *ART1* pathway that negatively regulate Al stress. The study of searching for major genes in the *ART1* control pathway has not been found comprehensively. This study tries to make rice cv. Al sensitive with a genome editing approach with the help of the Cas 9 protein. Al sensitive rice from cv. Inpago 5 aims to be a comparison in studying genes that encode repressors or negatively responsive genes to Al stress. The discovery of these new repressor genes can be a breakthrough in making Al-tolerant and abiotic-tolerant rice in the future.

There are four main transcription factors in plant tolerance to Al stress (Jingguang et al. 2020), i.e., *ART1*, *ASR1*, *ASR5*, and *OsWRKY22* (Fan et al. 2014; Jiang et al. 2017). The *ART1* transcription factor is reported to be a major gene related to Al tolerance. It can control other Al-responsive genes such as *OsMGT1* (Zhang et al. 2019), *OsCDT3*, *OsFRDL2* (Yokosho et al. 2016a), *OsFRDL4* (Yokosho et al. 2016b), *OsEXPA10*, *Nrat1* (Li et al. 2014; Xia et al. 2014), *OsALS1*, *STAR1*, and *STAR2* (Arbelaez et al. 2017). The ASR1 and ASR5 transcription factors can regulate Al tolerance genes, especially *Nrat1* (Arenhart et al. 2014, 2016). The *OsFRDL4* gene can be controlled by the *OsWRKY22*, ASR1, and *ASR5* transcription factors (Jingguang et al. 2020). Al tolerance genes have relatively different modes of action in regulating plant regulation to avoid Al stress (Jingguang et al. 2020). *OsFRDL4* is a gene that encodes a transporter related to the release of citrate into the rhizosphere. *Nrat1* is an Al transporter in the plasma membrane, and after Al enters the cytosol, putesin can inhibit Al (Jingguang et al. 2020). The *OsALS1* gene plays a role in accumulating Al in the vacuole, where its role is as a transporter in the tonoplast in rice (*O. sativa*) (Jingguang et al. 2020). The *OsMGT1* gene encodes a transporter whose role is to inhibit Al from entering the cytosol by having a high affinity for Mg ions compared to Al. The *OsALMT4* gene is reported to act as a malate transporter so that it can chelate Al in the rhizosphere (Liu et al. 2017). The *STAR1* and *STAR2* genes are genes related to the regulation of UDP-glucose, which is transported to the cell wall (Li et al. 2016; Zhu et al. 2019); therefore, it can prevent Al from entering the cell (Yang et al. 2017; Yan et al. 2018). Recent reports indicate that *OsPIN2* also plays a role in Al regulation (Adamowski and Friml 2015; Bai et al. 2017).

One of the latest genes reported to be related to Al tolerance is *OsGERLP* (Miftahudin et al. 2021). The *OsGERLP* is a gene that encodes a protein similar to ribosomal L32. Silencing of the *OsGERLP* gene has been reported to cause rice plants to become sensitive to Al. Conversely, overexpression of the *OsGERLP* gene has been reported to increase the level of plant tolerance to Al stress in both tobacco and transgenic rice (Miftahudin et al. 2021). The role of *OsGERLP* in plant tolerance to Al stress is also known to be regulated by acid or low pH (Miftahudin et al. 2021). However, the role and mechanism of the *OsGERLP* gene are still being investigated further, including its relationship with the *OsART1* gene.

Genetic engineering studies, particularly generating transgenic and/or mutant lines to understand abiotic stress, Al tolerance, and yield, are quite important (Guo and Ye 2014; Chen et al. 2017). The CRISPR/Cas 9 technique is a gene or genome editing technique (Agarwal et al. 2006) that is widely used in bacteria, plants (*O. sativa*, *Zea mays*, *Musa acuminata*, and others), animals, and fungi. In plants, this technique is widely used to study the regulation and mechanism of expression and interaction of a gene with other genes (Halim et al. 2021; Pratami et al. 2022). This technique is also used to make mutants sensitive to abiotic stress or to overexpress the promoter region controlling a particular gene. In this study, the construction of the recombinant pRGEB32-sgART1 vector was used to create Al-sensitive rice.

The *ART1* gene is a major transcription factor that plays a major role in plant tolerance to Al stress in japonica rice subspecies. The study and mechanism of Al stress tolerance in Indica rice are still very little studied. Random mutations that are non-homologous end joining (NHEJ) in the Cas9 protein (Halim et al. 2021) in the recombinant pRGEB32-sgART1 vector target the functional region of the *ART1* gene so that the resulting rice plant mutants express knock-down on *ART1*. In this study, recombinant *E. coli* was successfully transformed using the heat-shock-based transformation technique (Patigu et al. 2021). Construction of *E. coli* recombinant containing pRGEB32-Art1 was successfully generated (Figure 1). PCR-colony of the bacteria showed all *E. coli* had pRGEB32 recombinant. We found a 425 bp band of specific primers of selected regions of pRGEB32, both in the *E. coli* colony and in the plasmid (Figure 2). Therefore, transformation using heat shock in this method was precisely optimized. Optimization of transformation efficiency in *E. coli* depends on the efficiency and accuracy of the transformation method (Chan et al. 2013). The recombinant vector construction process successfully inserted sgRNA-ART1 into the pRGEB32 vector. Verification of the pRGEB32 insert in both recombinant *E. coli* and the recombinant vector showed the presence of a 425 bp band amplicon for the insert from the region between M13 reverse and sgRNA-ART1. The recombinant pRGEB32 plasmid sequence showed that the ART1 sgRNA was successfully inserted and inserted at a specific region at the *Bsa1* restriction site (Figure 3).

In conclusion, the construction of pRGEB32-sgART1 derived from *ART1* genomic DNA of rice cv. Inpago 5 in this study was successful. This success is demonstrated by several stages of the method and by the design of sgRNA-ART1, which was carried out in silico and in vitro.

ACKNOWLEDGEMENTS

We thank the Indonesian Endowment Fund for Education (LPDP/*Lembaga Pengelola Dana Pendidikan*) and the National Research and Innovation Agency (BRIN/ *Badan Riset dan Inovasi Nasional*), through number 152/IV/KS/11/2023, for their financial support of the *Riset dan Inovasi untuk Indonesia Maju Gelombang 4* (RIIM 4) research, especially for the Fiscal Year (FY) 2023-2025 grant. This work was also supported by funding from the Ministry of Education, Culture, Research, and Technology (number 0667/ES/AL.04/2024-5690) through the *Penelitian Fundamental Reguler* (PFR) scheme.

REFERENCES

Adamowski M, Friml J. 2015. PIN-dependent auxin transport: Action, regulation, and evolution. Plant Cell 27 (1): 20-32. DOI: 10.1105/tpc.114.134874.

Agarwal PK, Agarwal P, Reddy MK, Sopory SK. 2006. Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep 25: 1263-1274. DOI: 10.1007/s00299-006-0204-8.

Arbelaez JD, Maron LG, Jobe TO, Pineros MA, Famoso AN, Rebelo AR, Singh N, Ma QY, Fei ZJ, Kochian LV, McCouch SR. 2017. Aluminum Resistance Transcription Factor 1 (*ART1*) contributes to natural variation in aluminum resistance in diverse genetic backgrounds of rice (*O. sativa*). Plant Direct 1 (4): e00014. DOI: 10.1002/pld3.14.

Arenhart RA, Bai Y, de Oliveira LF, Neto LB, Schunemann M, Maraschin-Fdos S, Mariath J, Silverio A, Sachetto-Martins G, Margis R, Wang ZY, Margis-Pinheiro M. 2014. New insights into aluminum tolerance in rice: The ASR5 protein binds the STAR1 promoter and other aluminum-responsive genes. Mol Plant 7 (4): 709-721. DOI: 10.1093/mp/sst160.

Arenhart RA, Schunemann M, Bucker Neto L, Margis R, Wang ZY, Margis-Pinheiro M. 2016. Rice ASR1 and ASR5 are complementary transcription factors regulating aluminum-responsive genes. Plant Cell Environ 39 (3): 645-651. DOI: 10.1111/pce.12655.

Awasthi JP, Saha B, Panigrahi J, Yanase E, Koyama H, Panda SK. 2019. Redox balance, metabolic fingerprint, and physiological characterization in contrasting North East Indian rice for aluminum stress tolerance. Sci Rep 9 (1): 8681. DOI: 10.1038/s41598-019-45158-3.

Bai B, Bian HW, Zeng ZH, Hou N, Shi B, Wang JH, Zhu MY, Han N. 2017. miR393-mediated auxin signaling regulation is involved in root elongation inhibition in response to toxic aluminum stress in barley. Plant Cell Physiol 58 (3): 426-439. DOI: 10.1093/pcp/pcw211.

Chan WT, Verma CS, Lane DP, Gan SK. 2013. A comparison and optimization of methods and factors affecting the transformation of *Escherichia coli*. Biosci Rep 33: e00086. DOI: 10.1042/BSR20130098.

Che J, Tsutsui T, Yokosho K, Yamaji N, Ma JF. 2018. Functional characterization of an aluminum (Al)-inducible transcription factor, ART2, revealed a different pathway for Al tolerance in rice. New Phytol 220 (1): 209-218. DOI: 10.1111/nph.15252.

Chen J, Fan X, Qian K, Zhang Y, Song M, Liu Y, Xu G, Fan X. 2017. pOsNAR2.1:OsNAR2.1 expression enhances nitrogen uptake efficiency and grain yield in transgenic rice plants. Plant Biotechnol J 15 (10): 1273-1283. DOI: 10.1111/pbi.12714.

Fan W, Lou HQ, Gong YL, Liu MY, Wang ZQ, Yang JL, Zheng SJ. 2014. Identification of early Al-responsive genes in rice bean (*Vigna umbellata*) roots provides new clues to molecular mechanisms of Al toxicity and tolerance. Plant Cell Environ 37 (7): 1586-1597. DOI: 10.1111/pce.12258.

Fendiyanto MH, Hastilestari BR, Maysha DJ. 2023. *LCYB* gene expression and morphophysiological traits of *Musa acuminata* cultivars. SABRAO J Breed Genet 55 (6): 1984-1993.

Fendiyanto MH, Satrio RD, Junaedi A, Supena EDJ, Hairmansis A, Nugroho S, Miftahudin M. 2024. Correlation and path analyses for shoot architecture, photosynthesis, and yield-related traits in recombinant inbred lines of rice. SABRAO J Breed Genet 56 (4): 1609-1620.

Fendiyanto MH, Satrio RD, Pratami MP, Nikmah IA, Sari NIP, Widana IDKK, Darmadi D. 2021. Analysis of *superoxide dismutase* (*OsSOD*) gene expression using qRT-PCR, its morphophysiological characters, and path analysis in rice variety IR64 under aluminum stress. Intl J Agric Biol 26: 546-554. DOI: 10.17957/IJAB/15.1866.

Fendiyanto MH, Satrio RD, Suharsono S, Tjahjoleksono A, Hanarida I, Miftahudin M. 2019b. QTL for aluminum tolerance on rice chromosome 3 based on root length characters. SABRAO J Breed Genet 51 (4): 451-469.

Fendiyanto MH, Satrio RD, Suharsono S, Tjahjoleksono A, Miftahudin M. 2019a. Correlation among Snpb11 markers, root growth, and physiological characters of upland rice under aluminum stress. Biodiversitas 20 (5): 1243-1254. DOI: 10.13057/biodiv/d200514.

Guo LB, Ye GY. 2014. Use of major quantitative trait loci to improve grain yield of rice. Rice Sci 21 (2): 65-82. DOI: 10.1016/S1672-6308(13)60174-2.

Halim I, Fendiyanto MH, Miftahudin M. 2021. sgRNA design for *DLT* gene editing using CRISPR-Cas9 and in-silico mutation prediction in Rice cv. Hawara Bunar. IOP Conf Ser Eart Environ Sci 948 (1): 012083. DOI: 10.1088/1755-1315/948/1/012083.

Hou L, Zhang X, Li Y, Chen S, Qu H, Yu J, Zhang L, Fan Z. 2016. Rapid screening of recombinant plasmids by direct colony quantitative real-time PCR. Adv Biosci Biotechnol 7 (10): 428-433. DOI: 10.4236/abb.2016.710041.

Jiang J, Ma S, Ye N, Jiang M, Cao J, Zhang J. 2017. WRKY transcription factors in plant responses to stresses. J Integr Plant Biol 59 (2): 86-101. DOI: 10.1111/jipb.12513.

Jingguang C, Qi L, Baiquan Z, Longbiao G, Guoyou Y. 2020. Progress on molecular mechanism of aluminum resistance in rice. Rice Sci 27 (6): 454-467. DOI: 10.1016/j.rsci.2020.09.003.

Kang F, Wang H, Gao Y, Long J, Wang Q. 2013. Ca^{2+} promoted the low transformation efficiency of plasmid DNA exposed to PAH contaminants. PLoS ONE 8 (3): e58238. DOI: 10.1371/journal.pone.0058238.

Kochian LV, Hoekenga OA, Piñeros MA. 2004. How do crop plants tolerate acid soils? Mechanisms of aluminum toxicity and phosphorus efficiency. Annu Rev Plant Biol 55 (1): 459-463. DOI: 10.1146/annurev.arplant.55.031903.141655.

Kochian LV, Pi˜neros MA, Liu J, Magalhaes JV. 2015. Plant adaptation to acid soils: The molecular basis for crop aluminum resistance. Annu Rev Plant Biol 66: 571-598. DOI: 10.1146/annurev-arplant-043014-114822.

Kochian LV. 1995. Cellular mechanisms of aluminum toxicity and resistance in plants. Annu Rev Plant Biol 46: 237-260. DOI: 10.1146/annurev.pp.46.060195.001321.

Kusumawati A, Unsunnidhal L, Budiyanto A, Setyawan AMN, Gustari S. 2023. Construction of the pET-15b plasmid with the Jembrana disease virus tat gene. J Sains Vet 41 (1): 81-87. DOI: 10.22146/jsv.79217. [Indonesian]

Li GZ, Wang ZQ, Yokosho K, Ding B, Fan W, Gong QQ, Li GX, Wu YR, Yang JL, Ma JF, Zheng SJ. 2018. Transcription factor WRKY22 promotes aluminum tolerance via activation of *OsFRDL4* expression and enhancement of citrate secretion in rice (*Oryza sativa*). New Phytol 219 (1): 149-162. DOI: 10.1111/nph.15143.

Li JY, Liu JP, Dong DK, Jia XM, McCouch SR, Kochian LV. 2014. Natural variation underlies alterations in Nramp aluminum transporter (NRAT1) expression and function that play a key role in rice aluminum tolerance. Proc Natl Acad Sci USA 111 (17): 6503-6508. DOI: 10.1073/pnas.1318975111.

Li X, Sui X, Zhang Y, Sun Y, Zhao Y, Zhai Y, Wang Q. 2010. An improved calcium chloride method preparation and transformation of competent cells. Afr J Biotechnol 9 (50): 8549-8554. DOI: 10.5897/AJB10.105.

Li XW, Li YL, Qu M, Xiao HD, Feng YM, Liu JY, Wu LS, Yu M. 2016. Cell wall pectin and its methyl-esterification in transition zone determine Al resistance in cultivars of pea (*Pisum sativum*). Front Plant Sci 7: 39. DOI: 10.3389/fpls.2016.00039.

Liu J, Zhou MX, Delhaize E, Ryan PR. 2017. Altered expression of a malate-permeable anion channel, OsALMT4, disrupts mineral nutrition. Plant Physiol 175: 1745-1759. DOI: 10.1104/pp.17.01142.

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K. 1998. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain, separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 10 (8): 1391-1406. DOI: 10.1105/tpc.10.8.1391.

Ma JF, Chen ZC, Shen RF. 2014. Molecular mechanisms of Al tolerance in gramineous plants. Plant Soil 381: 1-12. DOI: 10.1007/s11104-014-2073-1.

Meng LJ, Wang BX, Zhao XQ, Ponce K, Qian Q, Ye GY. 2017. Association mapping of ferrous, zinc, and aluminum tolerance at the seedling stage in indica rice using MAGIC populations. Front Plant Sci 8: 1822. DOI: 10.3389/fpls.2017.01822.

Miftahudin M, Roslim DI, Fendiyanto MH, Satrio RD, Zulkifli A, Umaiyah EI, Chikmawati T, Sulistyaningsih YC, Suharsono S, Hartana A, Nguyen HT, Gustafson JP. 2021. OsGERLP: A novel aluminum tolerance rice gene isolated from a local cultivar in Indonesia. Plant Physiol Biochem 162: 86-99. DOI: 10.1016/j.plaphy.2021.02.019.

Miftahudin, Nurlaela, Juliarni. 2007. Uptake and distribution of aluminum in root apices of two rice varieties under aluminum stress. HAYATI J Biosci 14 (3): 110-114. DOI: 10.4308/hjb.14.3.110.

Miftahudin, Scholes GJ, Gustafson JP. 2002. AFLP markers tightly linked to the aluminum-tolerance gene *Alt3* in rye (*Secale cereal* L.). Theor Appl Genet 104: 626-631. DOI: 10.1007/s00122-001-0782-3.

Nguyen VT, Burow MD, Nguyen HT, Le BT, Le TD, Paterson AH. 2001. Molecular mapping of genes conferring aluminum tolerance in rice (*Oryza sativa* L.). Theor Appl Genet 102: 1002-1010. DOI: 10.1007/s001220000472.

Okonechnikov K, Golosova O, Fursov M. 2012. Unipro UGENE: A unified bioinformatics toolkit. Bioinformatics 28 (8): 1166-1167. DOI: 10.1093/bioinformatics/bts091.

Panja S, Saha S, Jana B, Tarakdas B. 2006. Role of membrane potential on artificial transformation of *E. coli* with plasmid DNA. J Biotechnol 127 (1): 14-20. DOI: 10.1016/j.jbiotec.2006.06.008.

Patigu RS, Wijayanti P, Sebastian A, Purwestri YA. 2021. Optimization of heat shock temperature and time on the transformation of pRGEB32 into *Escherichia coli* DH5α. J Biol Trop 21 (3): 632-640. DOI: 10.29303/jbt.v21i3.2811.

Pratami MP, Fendiyanto MH, Satrio RD, Nikmah IA, Awwanah M, Farah N, Sari NIP, Nurhadiyanta N. 2022. In-silico genome editing identification and functional protein change of *Chlamydomonas reinhardtii* Acetyl-CoA Carboxylase (CrACCase). Jordan J Biol Sci 15 (3): 431-440. DOI: 10.54319/jjbs/150312.

Saha I, Sarkar B, Ghosh A, De AK, Adak MK. 2019. Abscisic acid induced cellular responses of sub1A QTL to aluminium toxicity in rice (*Oryza sativa* L.). Ecotox Environ Safe 183: 109600. DOI: 10.1016/j.ecoenv.2019.109600.

Sakuma Y, Liu Q, Dubouzet JG, Abe H, Shinozaki K, Yamaguchi-Shinozaki K. 2002. DNA-binding specificity of the ERF/AP2 domain of *Arabidopsis* DREBs, transcription factors involved in dehydration- and cold-inducible gene expression. Biochem Biophys Res Comm 290 (3): 998-1009. DOI: 10.1006/bbrc.2001.6299.

Sambrook J, Fritsch ER, Maniatis T. 1989. Molecular Cloning: A Laboratory Manual. 2nd Ed. Cold Spring Harbor Laboratory Press, New York.

Satrio RD, Fendiyanto MH, Miftahudin M. 2024. Tools and techniques used at global scale through genomics, transcriptomics, proteomics, and metabolomics to investigate plant stress responses at the molecular level. In: Shahid M, Gaur R. (eds) Molecular Dynamics of Plant Stress and Its Management. Springer Nature, Singapore. DOI: 10.1007/978-981-97-1699-9_25.

Satrio RD, Fendiyanto MH, Suharsono S, Supena EDJ, Miftahudin M. 2021. Genome-wide SNP discovery, linkage mapping, and analysis of QTL for morpho-physiological traits in rice during vegetative stage under drought stress. Physiol Mol Biol Plants 27: 2635-2650. DOI: 10.17957/IJAB/15.1230.

Satrio RD, Fendiyanto MH, Suharsono S, Supena EDJ, Miftahudin. 2023. Mapping and identification of QTL for agro-physiological traits in rice (*Oryza sativa* L.) under drought stress. Plant Gene 33: 100397. DOI: 10.1016/j.plgene.2022.100397.

Satrio RD, Fendiyanto MH, Supena EDJ, Suharsono, Miftahudin M. 2019. Identification of drought-responsive regulatory genes by hierarchical selection of expressed sequence tags and their expression under drought stress in rice. Intl J Agric Biol 22: 1524-1532. DOI: 10.17957/IJAB/15.1230.

Sezonov G, Joseleau-Petit D, D'Ari R. 2007. *Escherichia coli* physiology in Luria-Bertani broth. J Bacteriol 189 (23): 8746-8749. DOI: 10.1128/JB.01368-07.

Si J, Zhou W, Fang Y, Zhou D, Gao Y, Yao Q, Shen X, Zhu C. 2023. Label-free detection of T4 polynucleotide kinase activity and inhibition via malachite green aptamer generated from ligation-triggered transcription. Biosensors 13 (4): 449. DOI: 10.3390/bios13040449.

Tanaka W, Yamauchi T, Tsuda K. 2023. Genetic basis controlling rice plant architecture and its modification for breeding. Breed Sci 73: 30-45. DOI: 10.1270/jsbbs.22088.

Tian XH, Li XP, Zhou HL, Zhang JS, Gong ZZ, Chen SY. 2005. *OsDREB4* genes in rice encode AP2-containing proteins that bind specifically to the dehydration-responsive element. J Int Plant Biol 47: 467-476. DOI: 10.1111/j.1744-7909.2005.00028.

Xia J X, Yamaji N, Che J, Shen RF, Ma JF. 2014. Differential expression of Nrat1 is responsible for Al-tolerance QTL on chromosome 2 in rice. J Exp Bot 65 (15): 4297- 4304. DOI: 10.1093/jxb/eru201.

Yamaji N, Huang CF, Nagao S, Yano M, Sato Y, Nagamura Y, Ma F. 2009. A zinc finger transcription factor ART1 regulates multiple genes implicated in aluminum tolerance in rice. Plant Cell 21 (10): 3339-3349. DOI: 10.1105/tpc.109.070771.

Yan L, Riaz M, Wu XW, Du CQ, Liu YL, Jiang CC. 2018. Ameliorative effects of boron on aluminum induced variations of cell wall cellulose and pectin components in trifoliate orange (*Poncirus trifoliate* (L.) Raf.) rootstock. Environ Pollut 240: 764-774. DOI: 10.1016/j.envpol.2018.05.022.

Yang ZB, He C, Ma Y, Herde M, Ding Z. 2017. Jasmonic acid enhances Al-induced root growth inhibition. Plant Physiol 173 (2): 1420-1433. DOI: 10.1104/pp.16.01756.

Yokosho K, Yamaji N, Fujii-Kashino M, Ma JF. 2016a. Functional analysis of a MATE gene OsFRDL2 revealed its involvement in Al-induced secretion of citrate, but a lower contribution to Al tolerance in rice. Plant Cell Physiol 57: 976-985. DOI: 10.1093/pcp/pcw026.

Yokosho K, Yamaji N, Fujii-Kashino M, Ma JF. 2016b. Retrotransposon-mediated aluminum tolerance through enhanced expression of the citrate transporter OsFRDL4. Plant Physiol 172 (4): 2327-2336. DOI: 10.1104/pp.16.01214.

Zhang L, Peng Y, Li J, Tian X, Chen Z. 2019. OsMGT1 confers resistance to magnesium deficiency by enhancing the import of Mg in rice. Intl J Mol Sci 20 (1): 207. DOI: 10.3390/ijms20010207.

Zhang MJ, Deng XP, Yin LN, Qi LY, Wang XY, Wang SW, Li HB. 2016. Regulation of galactolipid biosynthesis by overexpression of the rice MGD gene contributes to enhanced aluminum tolerance in tobacco. Front Plant Sci 7: 337. DOI: 10.3389/fpls.2016.00337.

Zhou J, Li X, Xia J, Wen Y, Zhou J, Yu Z, Tian B. 2018. The role of temperature and bivalent ions in preparing competent *Escherichia coli*. Biotech 8: 222. DOI: 10.1007/s13205-018-1243-x.

Zhu CQ, Cao XC, Bai ZG, Zhu LF, Hu WJ, Hu AY, Abliz B, Zhong C, Liang QD, Huang J, Zhang JH, Jin QY. 2019. Putrescine alleviates aluminum toxicity in rice (*Oryza sativa*) by reducing cell wall Al contents in an ethylene-dependent manner. Physiol Plant 167 (4): 471-487. DOI: 10.1111/ppl.12961.