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Dissertação apresentada ao Instituto de Biociências da Universidade de São Paulo, para a obtenção de Título de Mestre em Ciências, na Área de Botânica.

Orientador(a): Cristiane Paula Gomes Calixto

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DEDICATÓRIA

Dedico este trabalho à minha mãe, ao meu pai e à minha companheira, que tanto me apoiam no desenvolvimento da minha carreira profissional, e aos muitos, que assim como eu, dedicaram suas vidas à Ciência.

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RESUMO

O arroz é um dos alimentos mais consumidos no mundo, mas o estresse térmico afeta seu rendimento e qualidade dos grãos. Para identificar soluções mecanísticas que melhorem essa cultura diante das ameaças das mudanças climáticas, as respostas moleculares da termotolerância devem ser identificadas. O *splicing* alternativo de pré-mRNAs é um dos mecanismos envolvidos na resposta das plantas contra estresses, mas pouco se sabe sobre como a variação de isoformas transcritas é regulada por mudanças de temperatura. Os genes de arroz LOC_Os02g40900 e LOC_Os05g30140 são candidatos a reguladores de *splicing* sensíveis ao calor. Para investigar essa hipótese, foram gerados plasmídeos com base na metodologia CRISPR/Cas9 visando editar esses genes em plantas de arroz. Utilizando a estratégia de duplo direcionamento, foram escolhidas duas sequências-alvo na região codificante de cada gene. Essas sequências-alvo foram inseridas em vetores de expressão de plantas via PCR, subclonagem e Golden Gate Assembly. Os plasmídeos adequados foram confirmados por sequenciamento de Sanger e então inseridos em *Agrobacterium*, sendo confirmados por PCR de colônias. Para a transformação de plantas, calos de arroz foram obtidos induzindo a divisão de embriões indiferenciados em sementes de arroz. Após a indução e subcultivo dos calos, ocorreu a transformação via *Agrobacterium*, permitindo que os calos resistentes ao agente seletivo prosseguissem para o meio de regeneração, gerando plantas transgênicas, confirmadas por PCR e sequenciamento de Sanger para, pelo menos, uma das edições esperadas.

ABSTRACT

Rice is one of the most consumed foods in the world, but heat stress affects its yield and grain quality. To identify mechanistic solutions to improve this crop under climate change threats, the molecular responses of thermotolerance must be identified. Alternative pre-mRNA processing is one of the mechanisms involved in plant response against stresses, but little is known about how the variation of transcribed isoforms is regulated by temperature changes. The rice genes LOC_Os02g40900 and LOC_Os05g30140 are candidates for heat-sensitive splicing regulators. To investigate this hypothesis, it was generated plasmids based on the CRISPR/Cas9 methodology to knock out these genes in rice plants. Using the dual-targeting strategy, it was selected two target sequences in the coding region of each gene. These target sequences were inserted in plant expression vectors via PCR, sub-cloning, and Golden Gate Assembly. The correct plasmids were confirmed by Sanger sequencing and they were then inserted into *Agrobacterium* and confirmed by colony PCR. For plant transformation, rice calli were obtained by inducing undifferentiated embryo division in rice seeds. After calli induction and subculture, occurred the transformation via *Agrobacterium*, enabling that the calli resistant to the selective agent proceeded to the regeneration medium, generating transgenic plants, confirmed via PCR and Sanger sequencing for at least one of the expected editions.

I. INTRODUCTION

Rice (*Oryza sativa* L.) represents one of the most iconic crops on the planet. It belongs to the division of Angiosperms (Magnoliophyta), the subgroup of monocots, and the family Poaceae. This cereal was initially domesticated in the valley of the Yangtze River (present-day Southern China) approximately 4,000 years ago. Rice is one of the oldest continuously cultivated foods by humanity (Zhao & Piperno, 2000).

Rice is among the most important foods for the expanding human population (Gnanamanickam, 2009). Although rice occupies the second place by planted area, it serves as the most important food source for several countries, mainly in Southeast Asia. This commodity provides about a fifth of the daily energy needed by more than half of the global population (Gomez, 2001; Datta, 2004; International Rice Genome Project, 2005).

Rice is cultivated on all continents, except Antarctica. Asia stands out as the world's largest producer, with the equivalent of 89.9%, followed by the American continent, with 5.0%. In Latin America and the Caribbean, the production of 27.3 million tons of rice represents 3.5% of world production, with emphasis on Brazil, which participates with 43% of this production. In 2022, Brazil had a production of 10.788 million tons, corresponding to 1.618 million hectares of sown area in the 2021/2022 harvest, mostly concentrated in the state of Rio Grande do Sul (EMBRAPA, 2023).

Given its commercial importance, the *O. sativa* has been the focus of many researches with international cooperation. In the year of 1998, the International Rice Genome Sequence Project (IRGSP) was created, which brought together resources from ten countries for sequencing and studying the genome of rice (*O. sativa* L. ssp. *Japonica*) (International Rice Genome Project, 2005). Subsequently, in 2004, the Rice Annotation Project (RAP) was created to allow more efficient analysis of genomic information through the process of annotation of rice sequences (Rice Annotation Project Consortium, 2008). As a result of the draft (initial) assembly of the rice genomic sequences presented by the IRGSP, the rice genome size was estimated at 389 Mb, having a size of 260 Mb larger than the model plant *Arabidopsis thaliana* (L.) Heynh. Divided into 12

chromosomes, *O. sativa* L. ssp. *japonica* has a genome with 55,986 genes (International Rice Genome Project, 2005; Rice Annotation Project Consortium, 2008).

Rice not only has economic and cultural importance for human civilization but also emerges as a model cereal for molecular biology studies, due to several reasons. To name a few, it is a diploid species with a completely sequenced genome and the generation of transgenic plants is relatively easy compared to other cereals. Additionally, rice genes are structurally and functionally homologous to a high degree with the corresponding genes of other important grasses, such as sugarcane, maize, and wheat. As a result, the knowledge obtained from rice genes can be used in other similar crops (Shimamoto & Kyozuka, 2002). Several other biotechnological approaches are being adopted to increase the quality and quantity of rice production, as well as its resistance to pests, diseases, and environmental stresses. This desired yield increase will have to be achieved with less land, less water, less labor, and less fertilizer (Coudert *et al*., 2010).

The desired increase in tolerance to environmental factors, especially the climate, is due to the climate changes of anthropic origin in which the planet is undergoing. According to the IPCC (2021), in 2019, atmospheric concentrations of CO_2 were higher than at any time in at least 2 million years, and concentrations of CH_4 was higher than at any time in at least 800,000 years, with an increase of 47% for CO_2 and 156% for CH_4, when compared with the period before the year 1750. Both CH4 and CO2 are greenhouse gases, and they play a pivotal role in the accelerated increase in global surface temperatures observed since 1970 (IPCC, 2021).

Temperatures during the most recent decade (2011-2020) exceed those of the warmest periods within the last 6,500 years. Extremes of heat (including heat waves) have become more frequent and more intense on all landmasses since the 1950s, while extremes of cold have become less frequent, and it is undeniable that human-induced climate change is the main factor of these alterations (IPCC, 2021).

Plants have a specific range of temperatures in which the physiological functions are stable, being favorable for growth, development, and reproduction. Temperatures above this optimum range are defined as heat stress, which can cause irreversible damage, depending on the intensity, duration, and stage of plant development (Wahid *et al*., 2007). As a result, both vegetative and reproductive organs can be severely impacted (Tenorio *et al*., 2013; Arshad *et al*., 2017). In rice, the heat damage is more detrimental during the reproductive stage, because it may lead to complete sterility in rice, and an impaired grain-

filling process, manifesting as reductions in grain yields and lower seed quality, respectively (Cao *et al*., 2008; Shah *et al*., 2011; Tenorio *et al*., 2013; Arshad *et al*., 2017; Wu *et al*., 2021).

Heat stress triggers the expression of certain genes and metabolites production, these together can enhance the heat tolerance in the plant (Hasanuzzaman *et al*. 2013). According to Wahid *et al*. (2007), heat tolerance is usually defined as plants that can minimize the stress effects and produce acceptable economic yields at high temperatures. At the molecular level, heat tolerance processes depend on ion transporters, factors participating in signaling cascades, osmolytes, antioxidant defense system, as well as transcriptional and post-transcriptional controls. These vital processes collectively act to counteract the deleterious effects of stress (Rodríguez *et al*. 2005).

Post-transcriptional controls play a crucial role in altering gene expression, particularly through the splicing machinery. The coding region of approximately 80% of plant nuclear genes are intercalated by noncoding intervening sequences, known as introns (Reddy, 2001). Therefore, to generate mRNAs with a complete open reading frame, introns must be removed from most precursor mRNAs (pre-mRNAs), a process termed pre-mRNA splicing. This intricate process is orchestrated by the spliceosome, a multimegadalton ribonucleoprotein (RNP) complex. The core of more than 99% of all spliceosomes found in higher eukaryotes are composed of five small nuclear RNPs (snRNPs): U1, U2, U4/U6 and U5. Additionally, numerous accessory proteins, including splicing factors (SFs), are also involved in splicing. The catalytic core of the spliceosome involves a paired complex with U2 and U6 snRNAs for the major form and U12 and U6atac snRNAs for the minor variant (~0.3% of all spliceosomes in higher eukaryotes) (Ciavarella *et al.*, 2020). In this discussion, we will focus on the major spliceosome, referred to hereafter as the spliceosome. The dynamic nature of both the conformation and composition of the spliceosome enrich the splicing machinery with precision and flexibility (Will & Lührmann, 2011).

The spliceosome recognizes introns primarily through highly conserved and key cis-elements present in introns. The three most crucial elements are the GU dinucleotide at the 5' splice site (5’ss), the AG dinucleotide at the 3' splice site (3’ss) – both of which mark the start and end of an intron, respectively – and the branch point sequence (BPS), positioned within 50 bases upstream of the 3' ss (Nosková *et al.*, 2023). The core snRNPs

within the spliceosome perform the essential roles of recognizing these key cis-elements and catalyzing the two splicing transesterification reactions (Fig. 1).

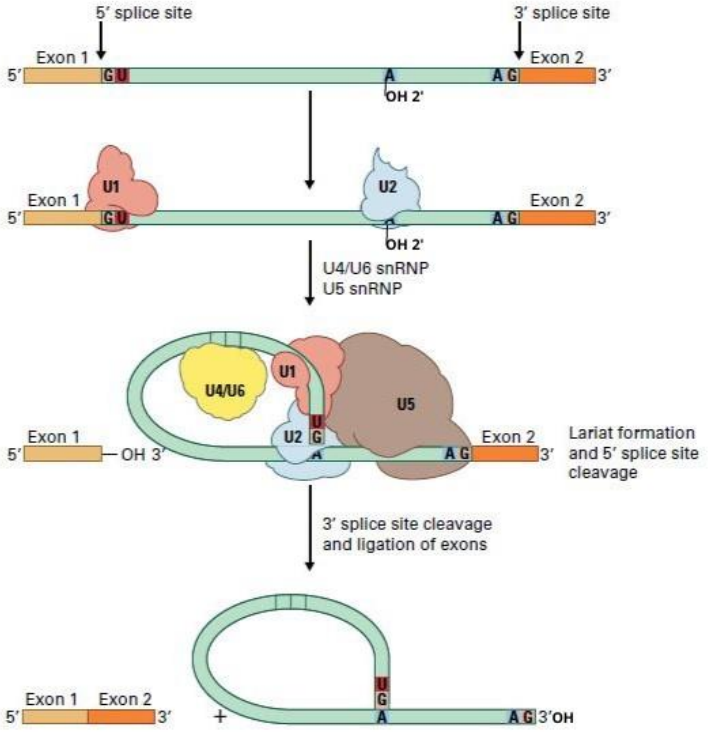

Figure 1. Simplified diagram of the nuclear pre-mRNA splicing. The splicing reaction starts with the nucleophilic attack of the 2′-hydroxyl group of the branch site adenosine on the 5′ splice site, creating the lariat intermediate and releasing the 5′ exon. Splicing concludes with a second nucleophilic attack by the 3′-hydroxyl group of the 5′ exon on the phosphodiester bond at the 3′ splice site, which connects the exons and releases the intron lariat (modified from Buchanan *et al*., 2015).

Since the seminal discovery of RNA splicing in 1977 by Berget *et al*. (1977), it has become evident that this process undergoes regulation in eukaryotes (Suran, 2020). A crucial family of proteins involved in splicing is the SR proteins, named for the domain with long repeats of serine (S) and arginine (R) amino acid residues. SR proteins are, a conserved example of splicing factors within eukaryotes (Plass *et al*., 2008). Once bound to the new pre-mRNA transcript, SR proteins stimulate the formation of the spliceosome, promoting the binding of U1 and U2 to the pre-mRNA, initiating spliceosome formation (Hastings & Krainer, 2001). SR proteins also aid U2 in recognizing and binding to the BPS, inducing spliceosome assembly at the specific 3' site selected by the SR proteins. Later in spliceosome formation, SR proteins assist in recruiting U4/U6 and U5 (Hastings & Krainer, 2001; Sanford *et al*., 2004).

The trans-acting splicing factors can influence the intron recognition process of the spliceosome (Kornblihtt *et al*., 2013). This interference has significant implications for gene expression, including the generation of different transcript isoforms from a single gene, a process known as alternative splicing (AS). Various types of AS events, such as intron retention, alternative exon, alternative 5'ss and/or 3'ss, among others, can lead to the generation of alternative transcripts. These AS events can affect transcripts in terms of subcellular localization, stability, function, and coding capacity. Notably, AS can be regulated by environmentally- or developmentally-driven alterations in the activity, localization, and/or levels of splicing factors, conferring an evolutionary advantage to gene expression regulation in eukaryotes (Kornblihtt *et al*., 2013).

In plants, AS emerges as a crucial regulatory layer in response to various external and internal cues (Syed *et al*., 2012). Recent genome-wide transcriptome mapping has revealed that plants exhibit a broad range of AS, spanning from 42% to 61% (Marquez *et al*., 2012; Reddy *et al*., 2013; Klepikova *et al*., 2016). Consequently, AS serves to reprogram gene expression, making it an important gene regulatory mechanism, especially in plant responses to environmental stresses (Calixto *et al*., 2016, 2018, 2019; Liu *et al*., 2021). For example, Verhage *et al*. (2017) have confirmed the crucial role of AS in adjusting to ambient temperature fluctuations by RNA-sequencing two *A. thaliana* accessions and a *Brassica* species subjected to varying temperatures. Notably, there was an enrichment of splicing-related genes within the pool of differentially alternatively spliced genes, indicating that genes from the splicing machinery itself undergo AS as temperatures change. These results suggest the involvement of splicing factors in the regulation of plant AS upon temperature change (Verhage *et al*. 2017).

Heat stress has been identified as a factor influencing splicing regulation. In a study by Chang *et al*. (2014) focusing on the moss species *Physcomitrella patens* (Hedw.) Bruch & Schimp., a basal lineage of land plants, it was observed that certain heat-sensitive alternative splicing (AS) events are conserved. This conservation implies that such regulatory mechanisms may have conferred advantages for plant survival during early evolution.

A more specific example of the importance of AS upon temperature changes was described in rice for the gene *OsDREB2B* (dehydration-responsive element-binding protein 2) (Matsukura *et al*., 2010). This gene has a dominant transcript under normal growth conditions, *OsDREB2B1*, that contains a 53-base pair exon 2 insertion. In plants

subjected to high-temperature stress, the isoform where exon 2 is spliced out (exon skipping), *OsDREB2B2*, becomes prevalent, losing the premature termination, so that the stress signal is transduced to a component of the splicing apparatus, and the splicing mechanism is changed to produce *OsDREB2B2*. This heat-induced AS response of *DREB2B* enables rice to rapidly produce the protein DREB2B in response to heat, independently of transcriptional activation (Matsukura *et al*., 2010). A similar AS mechanism has been identified in their respective orthologs in wheat, barley and maize (Xue & Loveridge, 2004; Egawa *et al*., 2006; Qin *et al*., 2007).

In rice, little is known about how the variation of transcribed isoforms is regulated by temperature changes. The levels and activity of hundreds of splicing factors change in response to temperature, suggesting an involvement of these genes in the regulation of alternative splicing of plants under heat-stress conditions (Calixto *et al*., 2018, 2019).

II. JUSTIFICATION

In crop plants, genetic erosion occurs through breeding practices. This loss of genetic diversity, and consequently phenotypic variation, is evident at both the species and gene levels due to selection and bottlenecks during domestication (Asano *et al*., 2011; Avni *et al*., 2017). This genetic erosion renders modern crop plants more vulnerable to stress, especially when compared with their wild relatives (Budak *et al*., 2015). To address this issue, plant breeders have employed various strategies to enhance diversity, such as mutagenesis, with the goal of creating novel genetic variants selected for improved yield and/or adaptability. However, this laborious and time-consuming method can lead to random, unintended, and potentially deleterious mutations, but they were ideal for starting research on gene function (Alonso & Ecker, 2006).

Several strategies have proven valuable in plant molecular biology for studying gene function. In mutagenesis procedures, many genes exhibit overlapping or redundant functions, requesting the isolation of multiple mutants to observe a loss-of-function phenotype. For instance, T-DNA transformation (Alonso *et al*., 2003) and Targeting Induced Local Lesions in Genomes (McCallum *et al*., 2000) have improved our understanding of the function and regulation of several genes. However, like traditional mutagenesis, these methods can introduce undesirable mutations. Consequently, large-scale screening of mutant populations becomes essential, rendering these methods costly, tedious, and inefficient (Alonso & Ecker, 2006). Antisense RNA (Hamilton & Baulcombe, 1999) and RNA interference (RNAi) (Hammond *et al*., 2001) have proven useful for disrupting the function of specific genes. However, these methods only indirectly or partially decrease gene function (Alonso & Ecker, 2006; Ma *et al*., 2016). While the techniques mentioned thus far have significantly contributed to our understanding of plant gene functions, their limitations lie in the lack of precision and cost-effectiveness for targeted mutagenesis and gene/genome editing.

The discovery of sequence-specific nucleases has enabled programmable gene editing, paving the way for precise genetic manipulation. These nucleases can be engineered to induce double-strand breaks at specific sites within the genome – breaks that can be repaired by the error-prone nonhomologous end-joining pathway, which frequently results in indels at the targeted sequence (Petolino, 2015). The currently most

widely used sequence-specific nuclease system is the clustered regularly interspaced short palindromic repeats/CRISPR/associated protein 9 (CRISPR/Cas9) (Jinek *et al*., 2012). The CRISPR/Cas9 system has been widely used as a gene editing tool for various organisms, including rice and many other plant species, enabling the generation of edited and non-transgenic plants (Minkenberg *et al*., 2017; Svitashev *et al*., 2016). The CRISPR/Cas9 complex (Fig. 2) uses engineered single-guide RNAs (sgRNAs) to target specific DNA sequences (Jinek *et al*., 2012), making it a simpler, more efficient tool than its predecessors.

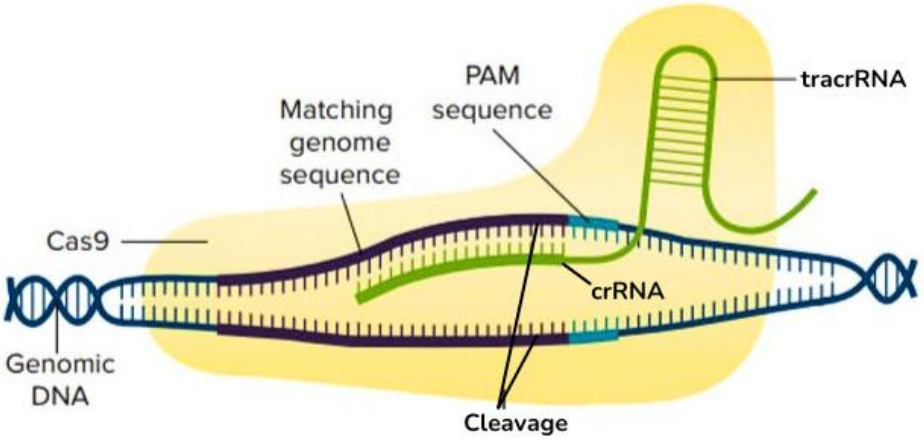

Figure 2. Scheme illustrating the mechanism of action of the CRISPR/Cas9 complex. The sgRNA, in green, denotes the crisprRNA (crRNA) in bold line, which is approximately 20 nucleotides specific to the gene of interest, and additionally includes a tracrRNA sequence that binds to the Cas9 enzyme. The PAM region of the target DNA (NGG) is depicted in light blue. Cleavage site is located three bases upstream of PAM region, creating a double-strand break. (Source: <www.moleculardevices.com/applications/gene-editing-with-crispr-engineering>).

Vitoriano & Calixto (2021) conducted an analysis of publicly available RNA-seq datasets to explore the extent of heat-induced alternative splicing (AS). Seeking to address the lack of comprehension regarding heat-sensitive AS regulators and the functions of alternative transcripts in plants, they identified over two thousand differentially spliced heat response genes, many of which were novel, including the LOC_Os02g40900 and LOC_Os05g30140 genes. Consequently, these genes were specifically selected for

investigation in this project, which involves the generation of knockout lines through CRISPR/Cas9-mediated editing.

The rice genes LOC_Os02g40900 and LOC_Os05g30140 (depicted in Fig. 3) appear to exhibit responsiveness to heat stress. The proteins encoded by these genes, particularly the alternative splice forms of LOC_Os05g30140 (LOC_Os05g30140.1 and LOC_Os05g30140.2), possess RNA recognition motifs. Additionally, these genes also respond to heat in many heat shock studies in rice (discussed in González-Schain *et al*., 2016). In this way, both genes are plausible candidates for regulating heat-sensitive processing. (Wilkins *et al*., 2016; Vitoriano & Calixto, 2021).

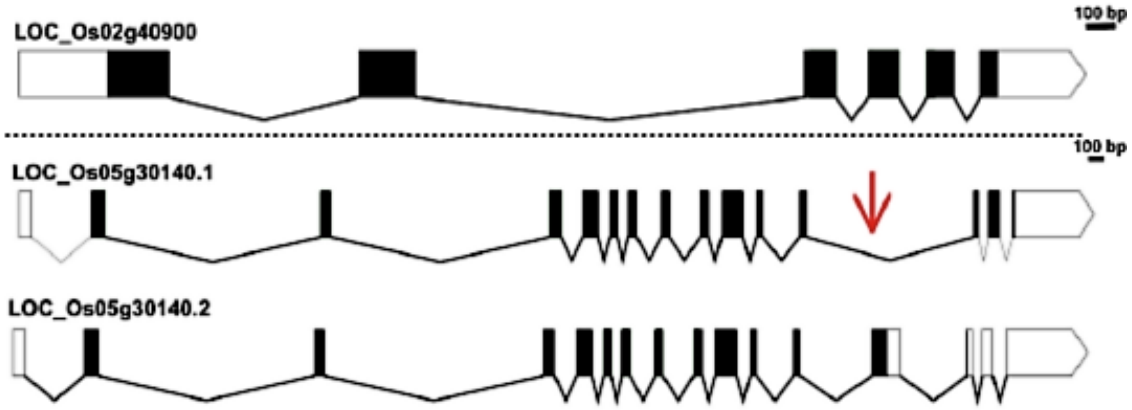

Figure 3: Structure of the transcript isoforms of LOC_Os02g40900 and LOC_Os05g30140. CDS length: 705 bp for LOC_Os02g40900, 885 bp for LOC_Os05g30140.1 and 864 bp for LOC_Os05g30140.2. Introns are represented by lines, Coding DNA Sequence (CDS) by dark boxes, and 5' and 3'- UTRs by white boxes. The position of the alternative splicing event is denoted by a red arrow. Image generated from Wormweb.org.

LOC_Os02g40900 and LOC_Os05g30140 share homology with crucial genes found in numerous models and economically significant plants. Specifically, LOC_Os02g40900 is orthologous to *At3g*04500 in *A. thaliana*, translated in a mRNA binding protein, and LOC_Os05g30140 is an ortholog of *At1g*55310 (*SCL33*), that encodes a spliceosome protein that interacts with U1 (TAIR database, 2023). These *A. thaliana* genes are known to exhibit substantial and rapid alterations in alternative splicing in response to temperature changes, as demonstrated by previous studies (Pajoro *et al*., 2017; Calixto *et al*., 2018). Furthermore, both rice genes are orthologous to genes that encode proteins featuring RNA recognition motifs (RRMs), also referred to as RNA-binding domains (RBDs) or ribonucleoprotein domains (RNPs). For LOC_Os02g40900,

the following orthologs genes express RRMs: *Bradi3g*12840 and *Bradi3g*49130 in *Brachypodium* sp., as well as *GRMZM2G016106* and *GRMZM2G125529* in *Zea mays* L.. On the other hand, LOC_Os05g30140 exhibits orthology to genes such as *Bradi2g*11990 in *Brachypodium* sp. and *GRMZM2G170365* in *Z. mays*, both of which specifically express ASF/SF2-like pre-mRNA splicing factors (Kawahara *et al*., 2013). These findings highlight the potential of both rice genes as regulators of heat-sensitive RNA processing.

The functional characterization of LOC_Os02g40900 and LOC_Os05g30140 has never been carried out. A brief analysis of their phylogenetic tree in the Plaza database (<https://bioinformatics.psb.ugent.be/plaza/>) revealed that none of these genes underwent a recent duplication event in rice (unpublish data of our group). This is an advantage for functional analysis, as it avoids the null phenotype of mutants due to functional redundancy, common in paralogs (Gu *et al*., 2003).

The use of the CRISPR/Cas9 technology to edit specific regions of the genome facilitates functional characterizations of several genes of interest. Notably, the CRISPR/Cas9 methodology was successfully employed to unravel the role of specific SFs and their contributions to splicing regulation and responses to abiotic stress (Butt *et al*., 2019; 2022; Kababji, 2019). For this reason, this work took advantage of this methodology and obtained rice plants containing CRISPR/Cas9 constructs that are able to edit two different splicing factors, LOC_Os02g40900 and LOC_Os05g30140. Transgenic plants were screened for genome edits able to knockout the genes of interest. Knockouts were selected and they will allow further investigation on the functionality of these genes of interest in a non-transgenic background, shedding light on the poorly understood aspects of SA regulation and its function in rice heat stress.

III. OBJECTIVES

3.1. MAIN OBJECTIVE:

Elucidation of the functions of LOC_Os02g40900 and LOC_Os05g30140 genes in rice.

3.2. SPECIFIC OBJECTIVES:

- To select and set up the guide sequences for the CRISPR/Cas9 gene-editing strategy;

- To clone and sequence the respective sequences of interest onto appropriate plasmids;

- To insert the sequences of interest into rice (*O. sativa* L. ssp. *japonica* cv. 'Nipponbare') via *Agrobacterium*;

- To select transgenic lines and their knockout lines for the genes of interest;

IV. MATERIAL AND METHODS

4.1. MATERIALS

4.1.1. Seed Bank

Rice plants were cultivated to obtain the seeds required to calli induction. Primarily, the seeds of cv. "Nipponbare" (cat. number BRA 00008226-3) were provided by "Empresa Brasileira de Pesquisa Agropecuária (Embrapa)" via a Material Transfer Agreement (MTA). The husk (palea and lemma) was manually removed, followed by sterilization in 70% ethanol for 1 minute with gentle mixing and subsequent treatment with 2-3% sodium hypochlorite for 45 minutes. The seeds were then washed five times with autoclaved distilled water in the laminar flow, and then were placed on sterile petri dishes containing filter paper moistened with distilled water. The sealed plates remained under a 12-hour photoperiod (Photon Flux Density [PFD] of 50 μmol/ m^{-2}/ s^{-1}) until seedling formation. Seedlings were transplanted into soil containing the compounds outlined in Table 1. An iron supplement (Kelamyth Fe 6% GREENHASTM) was applied every three weeks up until the flowering stage. The rice plants were kept into natural photoperiod until seed formation.

Table 1. Components of soil mix.

Component	Amount
PlantMax® substrate	5 L
Evidence® 700WG Insecticide Bayer™	1.5 mL
Forth Cote® N-P-K (15-09-12) Fertilizer	20 g

4.1.2. Vectors & Primers

The complete list of plasmid vectors (Table 2) and primers (Table 3) used in this work are listed below.

Table 2. Name and sequence length of plasmid vectors used in PCR reactions.

Plasmid name	Sequence length (bp)
pBract214m-Cas9-HSPT-FIGL1-FANCMgRNA/SpeI*	12.258
pBract214m-Cas9-HSPT-OsU6pgRNA/PaqCI*	11.710
pSoup*	9.274
pC95-AttRNA/EcoRI*	3.069
pJET 1.2/blunt	2.974

*Plasmids designed and provided by Dr Abdellah Barakate (James Hutton Institute – UK) via an MTA.

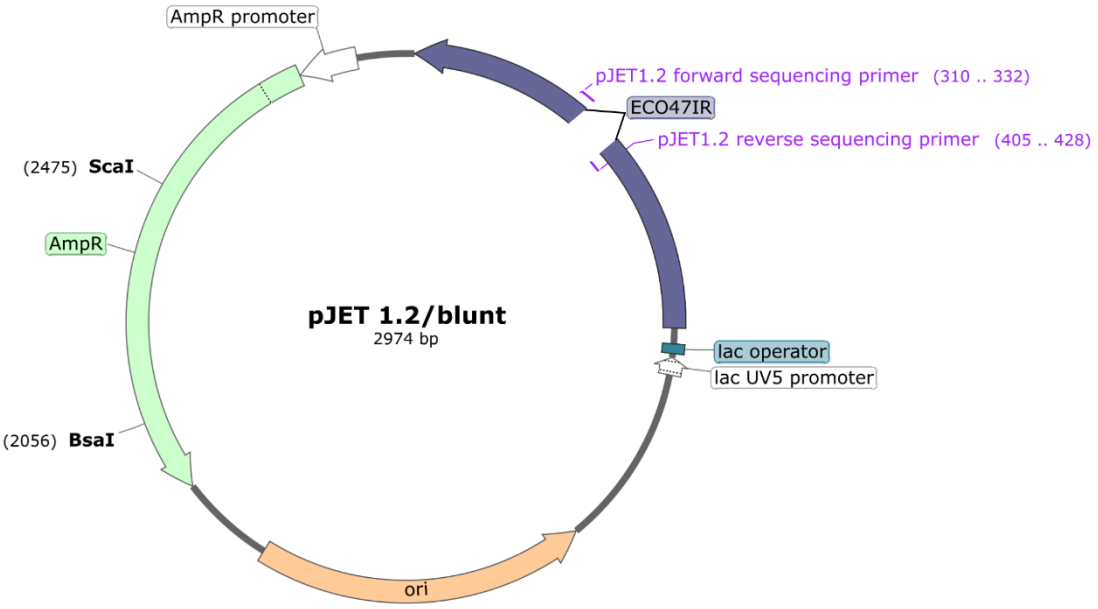

Figure 4. Map of the pJET 1.2/blunt vector. *AmpR*: gene that encodes the β-lactamase enzyme, which confers resistance to ampicillin; *ECO47IR*: lethal gene split by a blunt end site, which is where fragments are inserted during the ligation reaction; restriction sites for BsaI and ScaI enzymes; *Lac operator*: gene for expression of lactase; pJET forward and reverse primers. PCR Map generated by SnapGene® Viewer software (Insightful Science; available at snapgene.com).

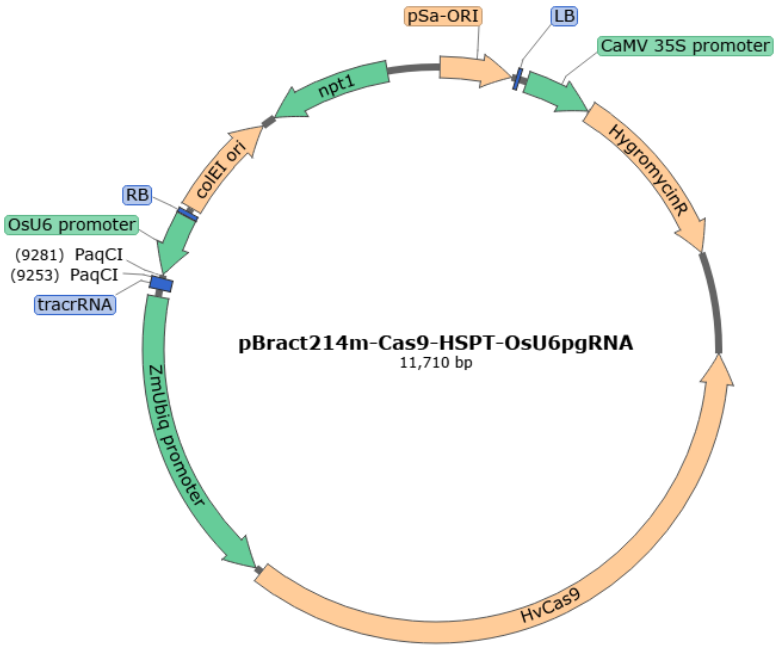

Figure 5. Map of the pBract214m-Cas9-HSPT-OsU6pgRNA vector showing the most relevant elements. In between the Left and Right Borders (LB and RB, respectively), the vector carries the *Cas9* gene, under the control of ZmUbiq promoter, the gene responsible for Hygromycin resistance, under the control of CaMv 35Spromoter, the OsU6 promoter, the tracrRNA, and the PaqCI sites at positions 9281 and 9253. Gene *npt1* was used as a bacterial selection marker. pSa-ORI: origin of replication in *Agrobacterium*. colEI ori: origin of replication in *E. coli*. Map generated by SnapGene® Viewer software (Insightful Science).

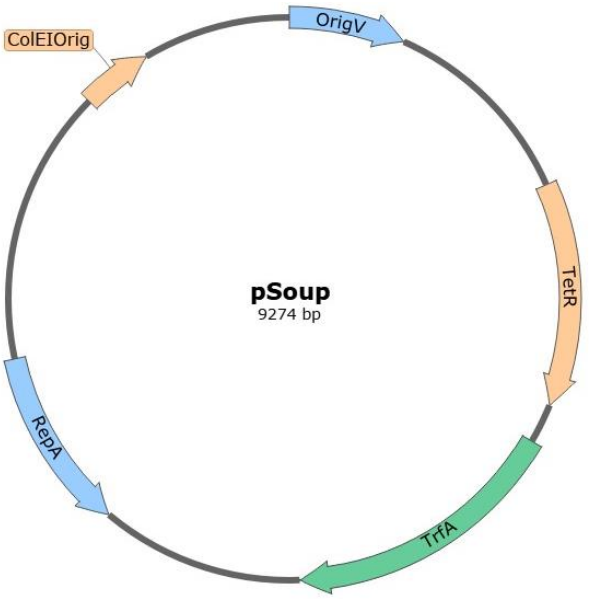

Figure 6. Map of the pSoup vector. ColEIOrig and OrigV: bacterial origin of replication. *RepA* and *TrfA* genes encodes a replication initiation protein. *TetR* gene reduces the repressor affinity for the tetracycline resistance gene (*TetA*) promoter operator sites. Map generated by SnapGene® Viewer software (Insightful Science).

Table 3. Name, sequence, and melting temperature (Tm) of primers used in PCR reactions.

Primer name	Sequence (5'-3')	Melting Temperature (°C)
02g40900_gRNA1	GAACACCTGCCCTTTGTTGTACGAGTACTGCGCGGAC GGTTTTAGAGCTAGAAATAGCAAG	81.1
02g40900_gRNA2	GAACACCTGCCCTTAAACTTCATCTCTTTCAATGCTGCAA CACAAGCGACAGCGCGCGGGT	84.1
05g30140_gRNA1	GAACACCTGCCCTTAAACCCACTTCCCTCTCGCGAATGTG CACCAGCC GGGAATCGAAC	84.9
05g30140_gRNA2	GAACACCTGCCCTTTGTTGCTAAACGTACTGATTACCGGT TTTAGAGCTAGAAATAGCAAG	78.3
gRNA-tRNA-F	AGTGGCACCGAGTCGGTGCAACAAAGCACCAGTGGTCTAG TG	81.2
gRNA-tRNA-R	CACTAGACCACTGGTGCTTTGTTGCACCGACTCGGTGCC ACT	81.2
pJET1.2_Fw	CGACTCACTATAGGGAGAGCGGC	65.2
pJET1.2_Rv	AAGAACATCGATTTTCCATGGCAG	60.5
Os05g30140_E5Fw	GCTAAACGTACTGATTACCG	59.0
ZmUbiqStartRv	CTGCAGAGGATCATACTTGTAACT	63.0
Os02g40900_Ex1Antisense	GTACGAGTACTGCGCGGACG	67.6
Os02g40900_Ex4Antisense	TTCATCTCTTTCAATGCTGC	59.6
Os02g40900_PromFw	GCCCGAACCGTGTCAAATAA	63.9
Os02g40900_Ex1Rv	CACGTTCTGGAGGTGGAAC	63.2
Os02g40900_3UTRv	GACGAGCCATACTACCCTAGA	63.1
Os05g30140_PromFw	TACATATCCTCCTCTCGCTCTC	63.2
Os05g30140_I2Rv	CGATGTACTCGCTGATACCAAA	63.1
Os05g30140_I9Rv	ACTGCTTGTCAGGTGTATCTTC	63.1

Calculated using the formula: 64.9 +41(yG+zC-16.4)/(wA+xT+yG+zC), where w, x, y, and z are the number of the bases A, T, G, C in the sequence, respectively.

The primers (Table 3) were designed using the PrimerQuest™ Tool from the Integrated DNA Technologies website (www.idtdna.com/pages/products/custom-dna-rna/dna-oligos). A BLASTn search was conducted for each primer against the NCBI nucleotide database to make sure there was no complementarity between the primers and other sequences within the rice genome. The forward and reverse primers were diluted in TE (10 mM Tris; 0.1 mM EDTA; pH 8) at a concentration of 100 **µ**M each for storage at – 20 ºC. Subsequently, 10 µl of the stock primers were diluted in 90 µl of Milli-Q water to obtain a working solution at a concentration of 10 **µ**M. The 10 **µ**M primers were then stored in a freezer at – 20 ºC.

4.2. sgRNAs & PLANT TRANSFORMATION VECTORS

4.2.1. sgRNA Construction

The sgRNAs construction was carried out using PCR and cloning through appropriate vectors. For this step, this work counted with the assistance of Dr. Barakate to design the most appropriate PCR and cloning strategies. Firstly, a search for targets in the CDS sequence was conducted using the web tool Crispr-P v2.0 (<www.crispr.hzau.edu.cn/cgi-bin/CRISPR2>), selecting the crRNA sequence starting with G for the U6 promoter and having the highest sgRNA on-target score and the lowest off-target score. U6, a type of RNA polymerase III promoter, is commonly utilized to express small RNAs like sgRNAs (Ma *et al*., 2014).

The U6 promoter has a requirement that the crRNAs sequences have to start with a guanine. Sequences of crRNAs that had a guanine as the first nucleobase base, as is the case for both crRNAs of the LOC_Os02g40900 gene, were cloned downstream of a U6 promoter -- one promoter for each crRNA. However, for the LOC_Os05g30140 gene, there was no second crRNA sequence that both started with a guanine and presented an on-score greater than 0.5. So, for this gene, a crRNA that did not start with a guanine but had an on-score > 0.5 was chosen, which led us to adopt the tRNA approach.

Two primers were designed to amplify the sgRNA constructs. The construct includes the AarI restriction site for subsequent ligation into the destination vector pBract214m-Cas9-HSPT-OsU6pgRNA/PaqCI. For the LOC_Os05g30140 gene, the tRNA approach required an additional two primers in the form of crRNA+tRNA, forming complete heterodimers. This strategy, therefore, involved a total of four primers, with both

sgRNAs connected by the tRNA spacer (sgRNA1-tRNA-sgRNA2) and positioned downstream to the U6 promoter.

In the amplification of sgRNAs, each PCR reaction was carried out with Phusion® DNA polymerase (Thermo Scientific), following the manufacturer's guidelines. (briefly shown in Table 4). To construct the sgRNAs aimed at gene LOC_Os02g40900, primers 02g40900_gRNA1 and 02g40900_gRNA2 were used, along with the plasmid pBract214m-Cas9-HSPT-FIGL1-FANCMgRNA/SpeI as the template (Fig. 4). As for sgRNAs for gene LOC_Os05g30140, three independent PCRs were performed (Fig 5. A-C). The first PCR used primers 05g30140_gRNA2 and gRNA-tRNA-R, with the linearized plasmid pBract214m-Cas9-HSPT-FIGL1-FANCMgRNA/SpeI as the template. The second reaction utilized 05g30140_gRNA1 and gRNA-tRNA-F, with the pC95-AttRNA/EcoRI linearized plasmid as the template. Lastly, the third and final reaction employed a mixture of each previous PCRs as a template, along with the primers 05g30140_gRNA1 and 05g30140_gRNA2. The cycle details used in the PCR followed the conditions specified in Table 5. PCR reactions were performed in the Applied Biosystems Veriti™ Dx 96-well Fast Thermal Cycler, 0.1 mL thermocycler.

Table 4. Components of the PCR using Phusion ® High-Fidelity DNA Polymerase.

Component	PCR concentrations
5X Phusion HF Buffer	4 ul
dNTP 10 µM	0.4 ul
Phusion DNA Polymerase	0.2 ul (1.0 units/50 µl PCR)
10 µM Forward Primer	1 ul
10 µM Reverse Primer	1 ul
DNA Template	0.5 ul
Nuclease-free water	q.s.p. 20 ul

Table 5. Thermocycling conditions for PCR using Phusion ® DNA Polymerase.

PCR Step	Time	Quantity of cycles
Initial Denaturation (98°C)	30 s	1
Denaturation (98°C)	5 s	30-35
Annealing/Extension (72°C)	15–30 s/kb	
Final Extension (72°C)	5 min	1

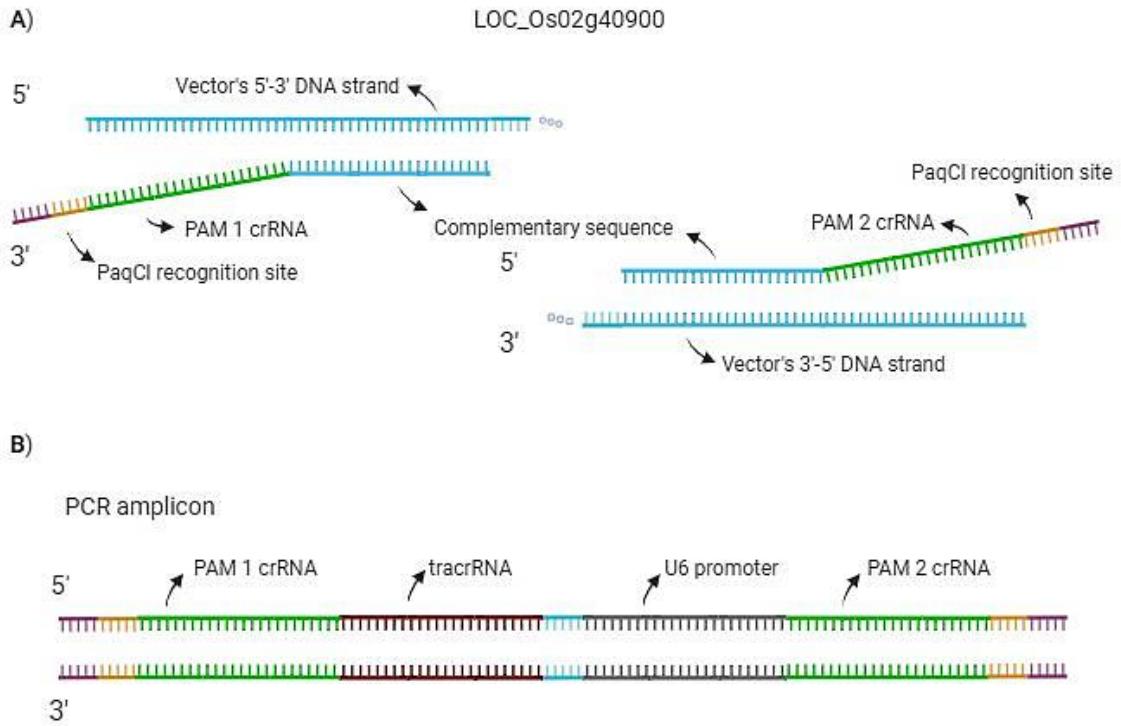

Figure 4. Strategy to obtain the LOC_Os02g40900 sgRNAs via PCR (A) and the final amplicon (B). Image created with BioRender.com.

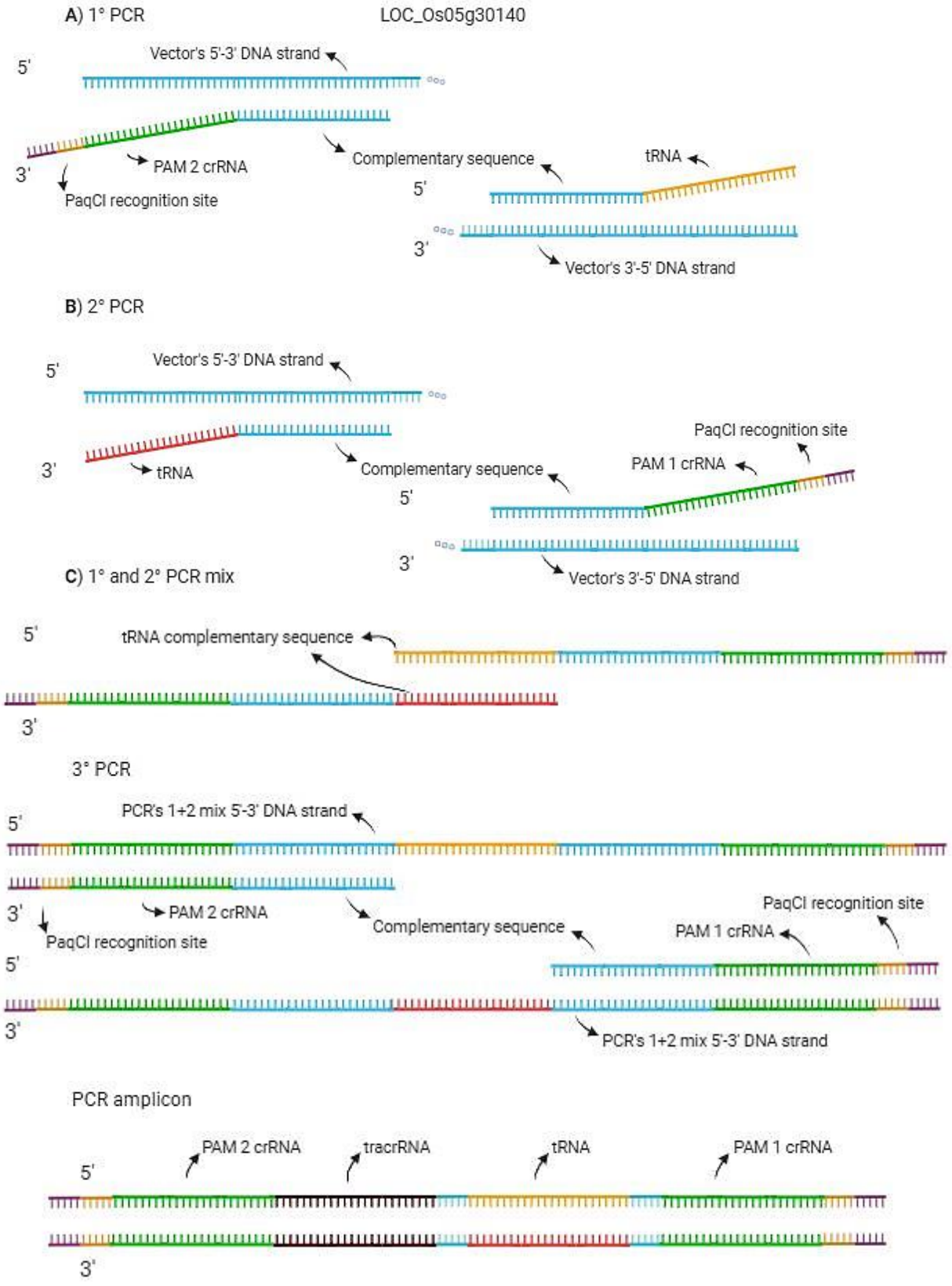

Figure 5. Strategy to obtain the LOC_Os05g30140 sgRNAs via PCR series (tRNA approach). A) First PCR. B) Second PCR. C) Third and last PCR, showing the mix of previous PCRs and the final amplicon. Image created with BioRender.com.

The products of each PCR reaction were analyzed by electrophoresis gel. The electrophoresis was conducted using a 1.7% agarose gel (UltraPure™ Agarose, Invitrogen), prepared by dissolving 0.85 g of agarose in 50 ml of Tris-Acetate-EDTA buffer (1X TAE). For the visualization of DNA bands, 2.5 µl of SYBR™ Safe DNA Gel Stain (Invitrogen) was added to the gel solution. Electrophoresis of each sample was carried out in a horizontal electrophoresis cell (Loccus LCH-7×8) at a voltage of 105 V and a current of 90 mA for a duration of 50 minutes.

4.2.2. DNA Extraction from Agarose Gel

DNA extraction from agarose gel was carried out with the NucleoSpin® PCR Clean-up kit following manufacturer's instructions. Briefly, the DNA fragment was excised from the gel using a clean scalpel to remove the excess agarose gel. The gel slice was transferred to clean microcentrifuge tube containing NTI buffer at a proportion of 1 ng of gel to 2 µl to NTI buffer, then it was incubated at 50 ºC for 10 minutes and vortexed in order to dissolve the gel. Then, each sample was placed in a NucleoSpin® Gel and PCR Clean-up Column. After centrifugation, the flow-through was discarded. Later, 700 µl of Buffer NT3 was added to each tube, followed by centrifugation and discarding the flow-through, repeated twice. To fully dry the silica membrane, it was centrifuged and placed at 70 °C for three minutes. Finally, in order to elute the DNA, 20 µl of NE buffer was added, followed by centrifugation. The concentration of the recovered DNA was performed using the Invitrogen Qubit 4 Fluorometer.

4.2.3. DNA Fragment Cloning

The PCR product of interest was inserted into the pJET 1.2/blunt vector (Fig. 4) (Thermo Scientific). This vector is designed for cloning PCR products synthesized by polymerases with proofreading activity that remove adenine overhangs at the 3' end of the synthesized strand. The blunt-end cloning reaction was carried out with the components listed in Table 6. The ligation reaction was incubated at room temperature for 5 minutes and subsequently used in the transformation step.

Table 6. Ligation reaction components.

Component	Amount
2X reaction buffer (Promega)	10 µl
target DNA (215-680bp)	100-200 ng
Vector pJET1.2/blunt	50 ng
T4 DNA ligase [0.1–1u] (Promega)	1 µl
Nuclease-free water to a final volume of	20 µl

4.2.4. *Escherichia coli* Transformation

E. coli transformation was carried out by adding 1 µl of the ligation reaction to an microcentrifuge tube containing 50 µl of electrocompetent *E. coli* cells, strain DH5α. The solution was then transferred to a Gene Pulser®/Micropulser™ (Bio-rad) cuvette and electroporated at 2.5 kV using the Micropulser™ (Bio-rad) electroporator. Immediately after electroporation, 1 ml of SOC culture medium (detailed in the <supplementary material>) was added to the cuvette. Subsequently, the contents of the cuvette were transferred to a test tube and incubated at 37 °C for 45 minutes at 180 RPM. Afterwards, 200 µl of the transformed bacteria was plated onto solid LB medium containing Ampicillin (100 mg/ml) (detailed in the <supplementary material>), which was then incubated at 37 °C overnight.

4.2.5. Plasmid Minipreparation

For the plasmid minipreparation, the initial step involved inoculating 1 µl of the chosen colony into test tubes containing 2 ml of LB medium and 2 µl of Ampicillin (100 mg/ml). These tubes were then placed in an incubator at 37°C and set to 180 RPM overnight. On the subsequent day, a portion of all the colonies was preserved by mixing 200 µl of each with 200 µl of autoclaved 50% glycerol, and the resulting stock was stored at -80°C.

Up to 1.5 ml of each bacterial sample was transferred to 1.5 ml microcentrifuge tubes and centrifuged at 14,000 RPM for 2 minutes. Subsequently, the supernatant solution was removed and 100 µl of Lysis Solution I (containing 50 mM glucose, 25 mM Tris-Cl at pH 8.0, and 10 mM EDTA at pH 8.0) was added. The pelleted cells were resuspended in this solution until no clumps remained. Afterward, 200 µl of Lysis Solution II (comprising 0.2 N NaOH and 1% (w/v) SDS) was added, and the tubes were gently

inverted before incubating them on ice for 5 minutes. Following the ice incubation, 150 µl of ice-cold Lysis Solution III (consisting of 60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, and 28.5 ml H2O) were added, and the tubes were gently inverted before another 5-minute incubation on ice.

After the ice incubation, the tubes were subjected to centrifugation at 14,000 RPM for 5 minutes, and the resulting supernatant was carefully transferred to new 1.5 ml tubes without disturbing the precipitate. To each sample, 400 µl of Isopropanol was added, followed by vortexing, a 2-minute room temperature incubation, and centrifugation at 14,000 RPM for 5 minutes. The supernatant was then discarded, and all tubes were left open at room temperature for 20 minutes to allow ethanol residues to dry. Finally, 40 µl of TE Buffer (containing 10 mM Tris, 0.1 mM EDTA at 1X, and pH 8) with 20 µg/ml of RNase was added to each sample. Final plasmid miniprep tubes were stored in a freezer at -20 °C.

4.2.6. Plasmid DNA Digestion

Plasmid digestion was carried out using appropriate restriction enzymes (10 U/µl Thermo Scientific). The digestion of each sample was prepared according to the components listed in Table 7. The samples were incubated at 37 ºC overnight, and then inactivated for standard period for each enzyme.

Table 7. Components of plasmid digestion.

Component	Volume
Restriction enzyme	0.5-1 µl
Plasmid DNA (1mg/ml)	17 µl
10X Buffer Mix	2 µl
Water to a final volume of	20 µl

4.2.7. Golden Gate Assembly

The insertion of each fragment of interest into a plant expression vector was accomplished through the Golden Gate Assembly® strategy (New England BioLabs or NEB™) (Table 8). This approach involved the use of the type two restriction enzyme PaqCI® (NEB™) and the T4 DNA ligase (NEB™) along with the additional components

detailed in Table 8. This reaction allowed for three key steps: 1) excision of the fragment of interest from the plasmid where it was present; 2) linearization of the pBract214m-Cas9-HSPT-OsU6pgRNA/PaqCI vector; and 3) ligation of the excised fragment into the linearized vector (Fig. 2). This reaction took place in a thermocycler, involving 30 cycles of 37 ºC for 1 minute, followed by 16 ºC for 1 minute, and then at 60 °C for 5 minutes to deactivate the enzymes. Finally, it was added an extra 1 ul of T4 DNA Ligase on each sample, and let over night at 4 ºC. The product of the ligation was inserted in *E. coli* DH10 through electroporation, and finally confirmed by colony PCR.

Table 8. Reaction components of the Golden Gate Assembly (NEB™).

Component	Volume
pBract214m-Cas9-HSPT-OsU6pgRNA (36 ng/ul)	2 µl
Plasmid DNA with fragment of interest (72 ng/ul)	1 µl
T4 DNA ligase Buffer (NEB™) (2x)	10 µl
PaqCI (NEB™) (10 mg/ml)	0.5 µl
PaqCI Activator (NEB™) (20 uM)	0.25 µl
MiliQ water to final volume of 20 µl.	5.25 µl

4.2.8. Colony PCR

Colony PCR was carried out using GoTaq® Flexi DNA Polymerase (Promega), along with other PCR components listed in Table 7. The template DNA was prepared by taking 1 isolated colony and diluting it in 100 µl of sterile Milli-Q water. The temperature settings for the thermocycler are provided in Table 5.

Table 9. Colony PCR components.

Component	Volume
5X Green GoTaq® flexi buffer	4 µl
dNTP mix (10 mM)	0.4 µl
$MgCl_2$ (25 mM)	2 µl
GoTaq® DNA polymerase (5u/µl)	0.15 µl
Fw primer (10 µM)	1.1 µl
Rv primer (10 µM)	1.1 µl
Colonies (template)	1 µl
Water to a final volume of	20 µl

4.2.9. *Agrobacterium* Transformation

The plasmids of interest were inserted, by electroporation, into *Agrobacterium tumefaciens* strain AGL1 containing the plasmid pSoup helper (Fig. 6). Briefly, LB medium supplemented with selection antibiotics was streaked from the glycerol stock of the respective electrocompetent AGL1 strain, and then an isolated colony was inoculated into the liquid medium. The desired plasmid, segregated by a minipreparation, along with the electrocompetent *Agrobacterium*, underwent electroporation and was subsequently incubated at 28 ºC for 3-4 days, and then stored in glycerol at -80 ºC.

4.3. RICE TRANSFORMATION cv. Nipponbare

The rice transformation protocol employed in this study was adapted from *Toki et al*. (2006) and Ozawa (2009). This procedure involves the genetic transformation of embryogenic callus cells derived from the Nipponbare cultivar via *Agrobacterium*. The transformed calli are then subjected to antibiotic selection, followed by regeneration, and the subsequent selection of transformed strains.

4.3.1. Calli Induction

The husk (palea and lemma) from rice seeds were manually removed, followed by sterilization in 70% ethanol for 1 minute with gentle mixing and subsequent treatment with 2-3% sodium hypochlorite for 45 minutes. The seeds were then washed five times with autoclaved distilled water in the laminar flow and dried on autoclaved filter paper. Next, every 10 seeds were distributed on a single plate containing N6D medium (Table 10) and incubated at 28 °C under a photoperiod of 12 hours of light (PFD of 50 μmol/ m^{-2}/ s^{-1}) and 12 hours of darkness for six to eight weeks to promote callus formation. After this period, each callus that was formed was individually transferred to NB medium (Table 10) and incubated in the dark at 28 °C for four weeks.

Table 10. Components and concentrations of the N6D and NB medium.

N6D	NB
Macro elements N6 (10x) – 100 mL/L	Macro elements N6 (10x) – 100 mL/L
Micro elements N6 (1000x) – 1 mL/L	Micro elements B5 (100x) – 10 mL/L
Vitamins N6 (100x) – 10 mL/L	Vitamins B5 (100x) – 10 mL/L
FeEDTA (10 mM) 100x – 10 mL/L	FeEDTA (10 mM) 100x – 20 mL/L
Myoinositol – 0.1 g/L	2,4-D (1 mg/ml) – 2 mL/L
Hydrolyzed casein – 0.3 g/L	Sucrose – 30 g/L
2,4-D (1 mg/ml) – 2 mL/L	Proline 0.5 g/L
Sucrose – 30 g/L	Glutamine – 0.5 g/L
Phytagel* – 3 g/L	Hydrolyzed casein – 0.3 g/L
	Phytagel* – 3 g/L

N6 Solutions (Chu, 1978). B5 Solutions (Gamborg, Miller & Ojima, 1968). * The pH of the solution was adjusted to 5.8-5.85 with 1M KOH before adding the Phytagel.

4.3.2. Calli & *Agrobacterium* Co-Cultivation

The sub-cultivation step increases the number of calli available. The calli were transferred to the NB medium (Table 9), and sealed with parafilm, then incubated in the dark at 28 °C, pricking out the calli every four weeks in the new NB medium.

Five days before transformation, the calli were transferred to a new NB medium. Four days before transformation, the *Agrobacterium* containing the appropriate construct of interest was streaked on LB agar containing Rifampicin (50 mg/L), Tetracycline (2.5 mg/L), and Kanamycin (100 mg/L) and incubated at 28 °C for three days. It was made a pre-inoculum by selecting one isolated colony in 10 ml of liquid LB containing the same concentration of antibiotics mentioned above. Next, 1 ml of this pre-inoculum was added to 50 ml of liquid LB containing the same concentration of antibiotics mentioned above, which was then incubated at 28 °C and 180 RPM, until it reached an optical density (OD 600) of 1 to 1.5. The inoculum was centrifuged at 4000 RPM for 5 minutes and the resulting pellet carefully resuspended in liquid NBA, with an OD 600 of 0.1 to 0.2. NBA is NB medium with Acetosyringone (100 mM), which is a molecule able to induce the virulence genes of *Agrobacterium* (Wang *et al*., 2019). After that, the calli were submerged in the suspension of NBA medium with *Agrobacterium* and incubated at 28 °C for 10 minutes, after which they were removed and dried on sterile filter paper to remove excess suspension. Next, each infected callus was incubated in a solid NBA medium in the dark at 25 °C for two days. After this period of co-cultivation, the calli were washed in sterile water containing the antibiotic Timentin (150 mg/L), four times for 10 minutes, or until the water became clear, then dried on the sterile filter. The calli

were placed on HTC medium, which is an NB media containing Hygromycin (50 mg/L), Timentin (150 mg/L), and Cefotaxin (200 mg/L) and incubated in the dark at 28 °C for three to five weeks. During the following days, if there was growth of *Agrobacterium*, the calli were transferred to new plates of HTC medium. In two or three weeks it was possible to obtain hygromycin-resistant calli, which were transferred to new plates of HTC medium and kept in the dark at 28°C for three to four weeks.

4.3.3. Calli Regeneration

Hygromycin-resistant calli were transferred to the regeneration medium (Table 11), and then incubated at 28 °C in 12 hours of light (PFD de 50 µmol/ m-2/ s-1) and 12 hours of darkness, for at least 30 days. Portions of the calli with roots and green shoots (seedling formation) were isolated and transferred to MS medium containing Hygromycin (50 mg/mL), and incubated at the same conditions for 10 to 14 days.

Table 11. Components and concentrations of the Ozawa's rice callus regeneration medium (Ozawa, 2009)

Ozawa's
MS Basal Medium – 4.4 g/L
Hydrolyzed Casein – 2 g/L
Sucrose – 30 g/L
Sorbitol – 20 g/L
Kinetin (10 mg/mL) – 250 µl
Naphthaleneacetic acid (NAA) - (10 mg/mL) – 10 µl
Phytagel* – 3 g/L

* The pH of the solution was adjusted to 5.8-5.85 with 1M KOH before adding the Phytagel

4.3.4. Calli Seedlings Cultivation on Soil

Seedlings grown in MS medium and selected in the presence of the antibiotic Hygromycin were transplanted into soil when they reached 10 cm high. The components to prepare the compost are outlined in Table 1. To maintain high humidity during the first week of soil adaptation, each plant was covered with a transparent plastic bag. They were grown until seed production in a greenhouse (natural photoperiod and sunlight). An iron supplement (Kelamyth Fe 6% GREENHAS^{TM}) was applied every three weeks up until the flowering stage.

4.3.5. Genomic DNA Extraction

The genomic DNA (gDNA) extraction was conducted using the "PureLink® Plant Total DNA Purification Kit" following the manufacturer's instructions. First, rice leaves were frozen in liquid nitrogen and ground to a powder. For every 100 mg of plant material, 250 µL of Resuspension Buffer (R2) was added and the mixture was vortexed until fully resuspended. Next, 15 µL of 20% SDS and 15 µL of RNase A (20 mg/mL) were added, followed by incubation at 55 °C for 15 minutes. The sample was then centrifuged at maximum speed (14,100 rpm) for five minutes. The lysate supernatant was transferred to a sterile microcentrifuge tube, and 100 µL of Precipitation Buffer (N2) was added. The samples were mixed and left to incubate on ice for five minutes before being centrifuged at maximum speed in a microcentrifuge for five minutes at room temperature.

Next, 250 µL of the lysate supernatant was transferred to a new sterile microcentrifuge tube and 375 µL of ethanol-containing Binding Buffer (B4) was added. The solutions were, mixed well, and the mixture was added to a PureLink® spin column, followed by centrifugation for 30 seconds at maximum speed. The flow-through was discarded, and the spin column was washed a few times with buffers provided in the kit. Then, 100 µL of Elution Buffer (E1) was added to the spin column, which was incubated at room temperature for one minute, and centrifuged at maximum speed for one minute. The eluted gDNA was stored at –20 °C.

The confirmation of transformed plants was conducted by PCR using the components listed in Table 5, with 0.5 µL of template gDNA. For the T-DNA designed to edit gene LOC_Os02g40900, the primers Os02g40900_Ex1Antisense and ZmUbiqStartRv were used. For the T-DNA designed to edit gene LOC_Os05g30140, the primers Os05g30140_E5Fw and ZmUbiqStartRv were used.

4.3.6. Deletion Confirmation of Target Genes

To investigate if any of the transgenic plants had a deletion in the genomic region of the targeted genes, PCRs were conducted. All reactions utilized gDNA extracted from transformed plants as templates, with appropriate primers (Table 1). For gene LOC_Os02g40900, the primer pair Os02g40900_PromFw and Os02g40900_3UTRv were utilized. For gene LOC_Os05g30140, primers Os05g30140_PromFw and Os05g30140_I9Rv were used. In case there was no deletion, another reaction aimed to

amplify the genomic region around PAM1 of each target gene for Sanger sequencing. For gene LOC_Os02g40900, the primer pair Os02g40900_PromFw and Os02g40900_Ex1Rv were used, while for gene LOC_Os05g30140, primers Os05g30140_PromFw and Os05g30140_I2Rv.

4.4. TEXT PROOFREADING

ChatGPT (<https://chat.openai.com>), developed by OpenAI™, provided valuable support in proofreading this work.

V. RESULTS & DISCUSSION

5.1. sgRNA CLONING

Before reporting on the results of the sgRNA cloning strategies, it is important to mention that the U6 promoter was chosen for the sgRNAs expression in plants. Transcription from this promoter is more efficient when starting with a guanine (personal communication with Dr. Barakate). Therefore, the two sequences of crRNAs chosen for the LOC_Os02g40900 gene followed this criterion (Table 12 and Fig. 6) and each could be individually inserted downstream of their U6 promoter. In the case of the LOC_Os05g30140 gene, one crRNA started with a guanine, while no optimal second crRNA sequence was identified, namely beginning with a guanine and having an on-score greater than 0.5. Consequently, for this gene, a crRNA that did not start with a guanine but exhibited an on-score > 0.5 was chosen, leading to the adoption of the tRNA approach. In this approach, the sgRNA1-tRNA-sgRNA2 sequence (arranged in tandem) is cloned downstream of a U6 promoter. The expression of this construct in plant cells will lead to the cleavage of the tRNA by the endogenous tRNA biosynthesis system, generating two separate sgRNAs. Xie *et al*. (2015) detailed in their study how the endogenous tRNA-processing system could enhance the CRISPR/Cas9 methodology. It was demonstrated that synthetic genes with a tandemly arrayed tRNA–gRNA architecture were precisely processed into sgRNAs with desired targeting sequences (crRNA) in vivo. These sgRNAs directed Cas9 to edit multiple chromosomal targets with an efficiency of up to 100%. This approach could be widely applied to enhance Cas9 targeting capability, overcoming limitations such as the one demonstrated here when designing crRNAs for gene LOC_Os05g30140.

Table 12. Specifications of the pair of crRNAs chosen for each gene of interest.

Gene name	PAM number	crRNA + PAM Region	ON-SCORE	OFF-SCORE
LOC_Os02g40900	1	GTACGAGTACTGCGCGGACGCGG	0.844	0.222
	2	GCAGCATTGAAAGAGATGAATGG	0.628	0.058
LOC_Os05g30140	1	CATTCGCGAGAGGGAAGTGGAGG	0.572	0.234
	2	GCTAAACGTACTGATTACCGAGG	0.895	0.100

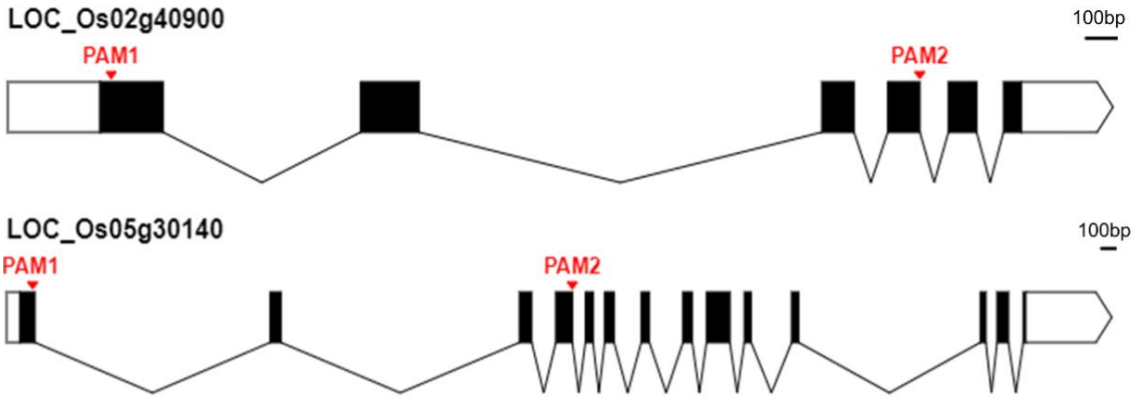

Figure 6. Structure of both target genes with location of selected PAMs regions for Cas9 protein recognition in the dual-targeting strategy. Introns are represented by lines, CDS by dark boxes, 5' and 3'- UTRs by white boxes. Image generated from Wormweb.org.

To obtain the sgRNA fragments containing the chosen crRNAs, different PCR strategies were employed for each gene and are detailed in the Material and Methods section 4.2.1. In the case of LOC_Os02g40900, a single PCR reaction was conducted, yielding the 608 bp expected band, while for LOC_Os05g30140, the third and last PCR reaction produced the 229 bp expected band (Fig. 7).

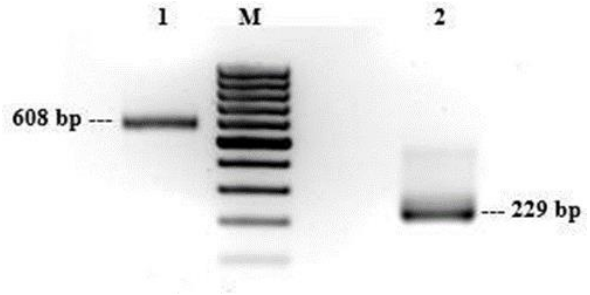

Figure 7. PCR fragments of the sgRNA construction for genes LOC_Os02g40900 (lane one) and LOC_Os05g30140 (lane two). Expected fragment sizes are 608 bp and 229 bp, respectively. M: GeneRuler™ 100 bp DNA ladder (Thermo Scientific™). 1.7% agarose gel.

For each gene of interest, the expected band was purified from the gel and ligated into the pJET 1.2/blunt vector, which may occur in the sense sequence or in its reverse-complement counterpart. *E. coli* cells were independently transformed with the two ligations, generating isolated colonies. Ten colonies were selected for each target gene, and subjected to plasmid minipreparation. For the LOC_Os02g40900 construct, four positive plasmids were confirmed through DNA digestion using the ScaI restriction enzyme, which showed expected fragments of sizes 2.686 bp and 896 bp (data not show).

For the LOC_Os05g30140 construct, BsaI and SmaI were used, enabling the selection of positive colonies (Fig. 8). Lastly, Sanger sequencing of two of the positive plasmids for each target gene was conducted, confirming that colonies 07 (LOC_Os02g40900) and 03 (LOC_Os05g30140) correctly contained the expected constructs, which were then named pOs02gJHS and pOs05gJHS (Fig. 9), respectively.

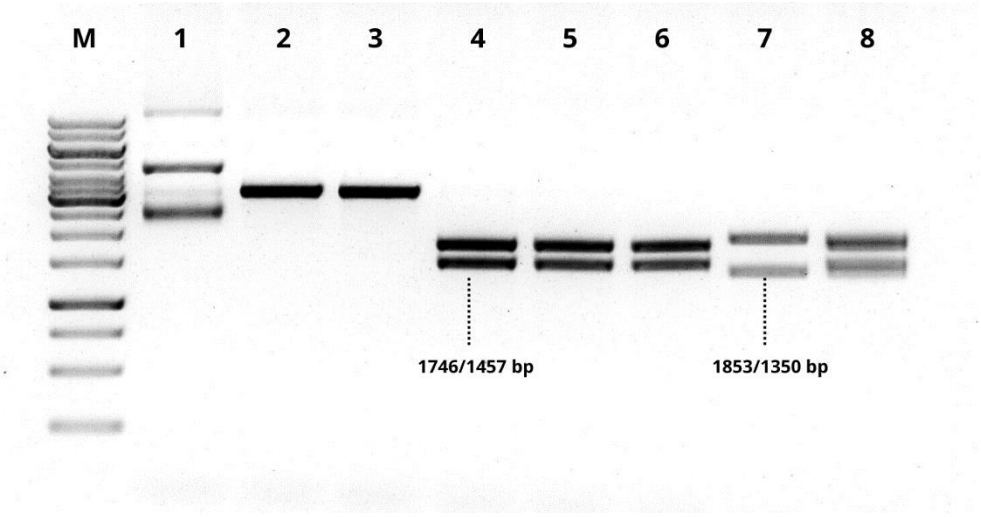

Figure 8. Digestion product for pOs05gJHS. Undigested plasmid 01 (lanes one); digested plasmid 01 with BsaI only (lane two); digested plasmid 01 with SmaI only (lane three); digested plasmids (01 to 05) with BsaI and SmaI restriction enzymes, with expected bands of 1746 bp and 1457 bp (lanes four to six, and eight), or bands of 1853 bp and 1350 bp (lane seven). M: GeneRuler™ 1 kb DNA ladder (Thermo Scientific™). 1.5% agarose gel.

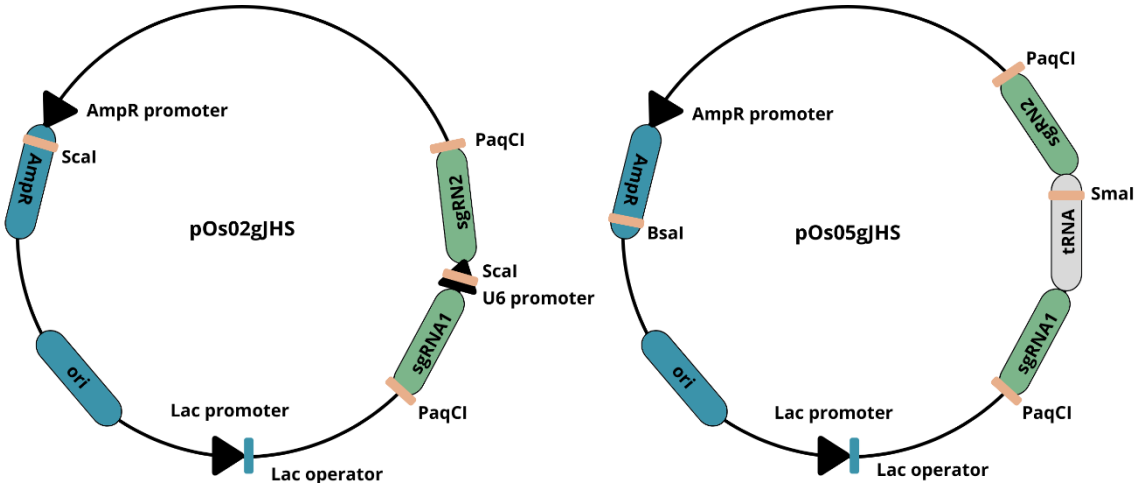

Figure 9. Map of confirmed plasmids pOs02gJHS and pOs05gJHS. *AmpR*: gene that encodes the β-lactamase enzyme, which confers resistance to ampicillin; ori as replication origin; *Lac operator*: gene for

expression of lactase; restriction sites for ScaI (pOs02gJHS only), BsaI and SmaI (pOs05gJHS only) and PaqCI enzymes.

5.2. PLANT EXPRESSION VECTORS

The insertion of each sgRNA into a plant expression vector was accomplished through the Golden Gate Assembly strategy (Fig. 10). It is worth noting that the plant expression vector also included the Cas9 enzyme gene and the gene for plant hygromycin resistance, serving as the in vitro selective agent. Following the Golden Gate Assembly reaction and transformation in *E. coli*, positive colonies were identified using colony PCR (Fig. 11). For the LOC_Os02g40900 gene, the primers Os02g40900_Ex1Antisense (forward) and ZmUbiqStartRv (reverse) were employed, producing the expected 701 bp fragment in four positive colonies. For the LOC_Os05g30140 gene, the primers Os05g30140_E5Fw (forward) and ZmUbiqStartRv were used, resulting in the expected 322 bp fragment in seven positive colonies. Subsequently, Sanger sequencing of two colonies for the gene LOC_Os02g40900 and two colonies for the LOC_Os05g30140 gene was conducted to confirm the presence of the fragments of interest for each target gene. In the case of LOC_Os02g40900, positive colony 06 underwent minipreparation of the pOsCas9-02gJHS plasmid, and the same procedure was applied to the pOsCas9-05gJHS plasmid from positive colony 17 for LOC_Os05g30140, making both plasmids suitable for *Agrobacterium* transformation.

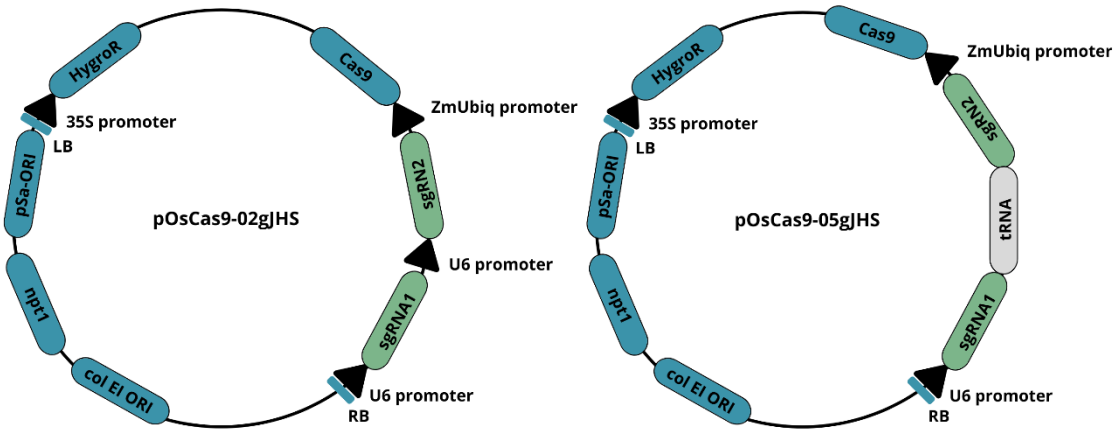

Figure 10. Map of confirmed plasmids pOsCas9-02gJHS and pOsCas9-05gJHS. The plasmids hold the backbone of pBract214m-Cas9-HSPT-OsU6pgRNA (Fig. 5) elements plus the fragments for each target gene.

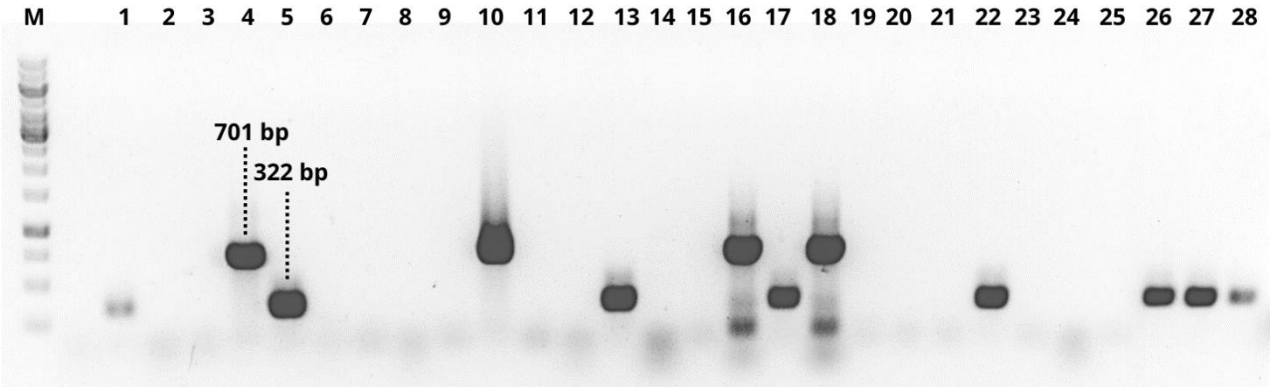

Figure 11. Colony PCR to confirm the presence of expected plant expression vectors. Colonies 03, 06, 09 and 10 (lanes four, ten, sixteen and eighteen, respectively), with expected band of 701bp for the gene LOC_Os02g40900. Colonies 04, 08, 11, 16, 17, 18 and 20 (lanes five, thirteen, seventeen, twenty-two, twenty-six to twenty-eight, respectively), with expected band of 322 bp for the LOC_Os05g30140 gene. Remaining lanes represent the negative colonies. M: GeneRuler™ 1 kb DNA ladder (Thermo Scientific™). 1.5% agarose gel.

Positive plasmids procedure to *Agrobacterium* transformations step. *Agrobacterium* strain AGL1 plus pSoup were independently transformed with the two desired plasmids (pOsCas9-02gJHS and pOsCas9-05gJHS), generating isolated colonies, and conferring resistance to tetracycline, rifampicin, and kanamycin antibiotics. For LOC_Os02g40900 gene, four positive colonies were identified through colony PCR using the primer pairs Os02g40900_Ex1Antisense and Os02g40900_Ex4Antisense, resulting in a 572 bp expected fragment (Fig. 12). Meanwhile, for LOC_Os05g30140 gene, the previously described Os05g30140_E5Fw and ZmUbiqStartRv primers were used, resulting in a 322 bp expected fragment in two positive colonies (Fig. 13).

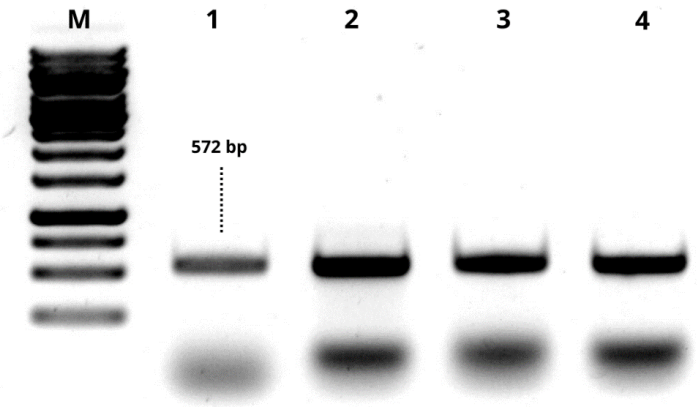

Figure 12. AGL1/pSoup *Agrobacterium* colony PCR to confirm the presence of pOsCas9-02gJH.. Colonies 01 to 04 (lanes one to four), with expected band of 572bp. M: GeneRuler™ 1 kb DNA ladder (Thermo Scientific™). 1.5% agarose gel.

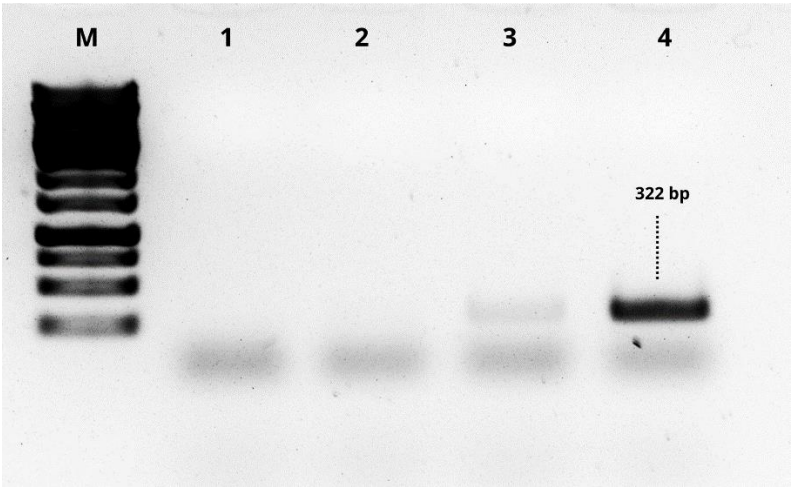

Figure 13. AGL1/pSoup *Agrobacterium* colony PCR to confirm the presence of pOsCas9-05gJHS. Colonies 03 and 04 (lanes three and four), with expected band of 322bp. Remaining lanes represent the negative colonies. M: GeneRuler™ 1 kb DNA ladder (Thermo Scientific™). 1.5% agarose gel.

5.3. PLANT TRANSFORMATION

At the beginning of this project, the lab of Prof. Calixto had no prior experience in rice transformation, and this method had to be developed for the first time. For this purpose, we received assistance from the working groups of Dr. Márcia Márgis and Dr. Felipe Ricachenevsky (UFRGS). Following this protocol, the calli required for genetic transformation were obtained by inducing undifferentiated embryo division in selected seeds through growth in N6D callus induction medium (Fig. 14. A & B). Six weeks after the initiation of induction (Fig. 14. C), several calli were obtained, and each healthy one was individually transferred to new plates containing NB medium for subculture and subsequent callus division (Fig. 14. D). After four weeks in the NB medium, the calli were ready to be genetically transformed by *Agrobacterium*. The process of callus induction was repeated every month, ensuring a continuous supply of fresh batches of healthy calli ready for transformation.

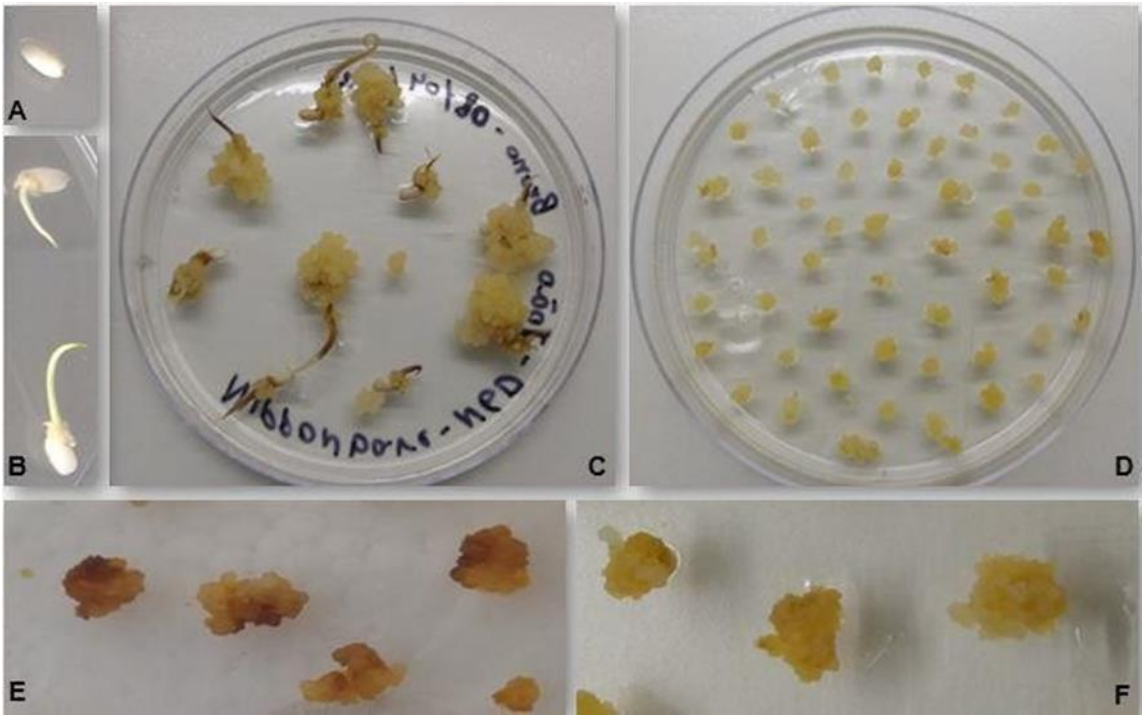

Figure 14. Rice calli induction and pricking out stages (cv. Nipponbare). Seeds after 1 day (A) and 1 week (B) in the callus induction medium (N6D). C) Calli with 04 weeks in induction medium and ready for transfer to the subculture medium (NB); D) Calli pricked out and suitable for co-cultivation with *Agrobacterium*; E) Calli after co-cultivation showing absence of Hygromycin resistance; F) Healthy calli after co- cultivation showing resistance to Hygromycin.

Co-cultivation is a crucial step for the success of calli transformation. During this process, the calli were cultured with transformed Agrobacterium in NB medium plus acetosyringone to induce *Agrobacterium* virulence. After two days at 25ºC in total darkness, transformed calli were washed with appropriate antibiotics to completely eliminate the *Agrobacterium*. The washed calli were plated for 30 days in NB medium containing hygromycin as the selective agent and antibiotics as an antibacterial agent, all in total darkness. To reduce the need for extensive washing due to the reappearance of *Agrobacterium* in the calli, we began using sterilized filter paper moistened with liquid NB on top of solid medium in the plates. This significantly decreased callus washing after co-cultivation, promoting the maintenance of potentially transformed cells and minimizing osmotic and mechanical stress resulting from washing. Resistant calli, maintaining their yellow color and not turning brown (Fig. 14. E & F), proceeded to the regeneration step.

The conditions for the regeneration step were meticulously determined in this study. Briefly, it involved defining the best growth medium and specifying suitable lighting and temperature conditions. Our investigations revealed that between the RHT50 medium and the one described by Ozawa (with some modifications), the latter proved to be the only one suitable for the regeneration stage, with none of the calli regenerated in the RHT50 medium. Non-transformed calli coming directly from the subculture stage went through the regeneration phase on the Ozawa medium. A remarkable success rate was achieved, regenerating a total of 30% of calli seedlings (per induced seed).

Several potentially transformed plants were obtained after the regeneration phase (Fig. 15). For the LOC_Os02g40900 construct, the calli regenerated a total of 21 plants from 14 different calli (example on Fig. 15. A). For the LOC_Os05g30140 construct, a total of seven plants from five calli were obtained. These plants were transferred to MS medium (example Fig. 15. B and C) supplemented with Hygromycin. After 10 days, the selected plants were transferred to soil (Fig. 16). For the LOC_Os02g40900 construct, a total of 12 plants from eight different calli (Os02g1.1; 1.2; 2.1; 3.2; 4.1; 5.1; 8.1; 9.1; 10.1; 10.2; 10.3; 10.4) successfully adapted to soil conditions. For the LOC_Os05g30140 construct, a total of five plants from four calli (Os05g1.2; 3.1; 4.1; 4.2; 5.1) were successfully transferred to soil.

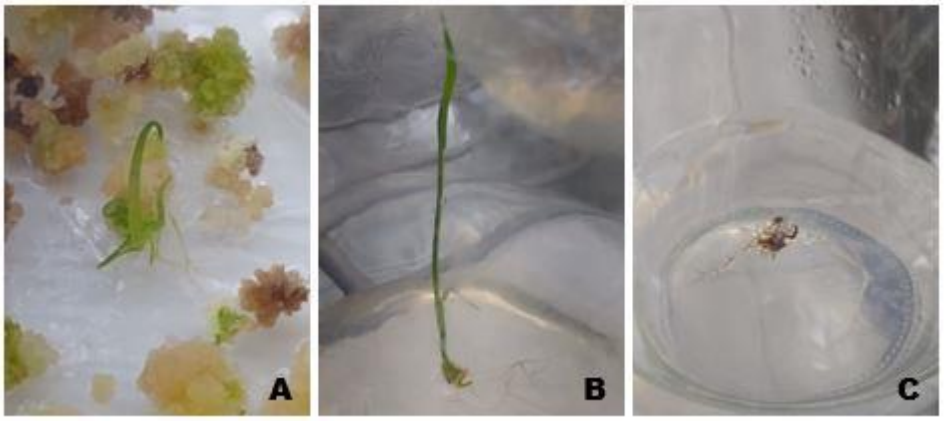

Figure 15. Rice callus regeneration stages (cv. Nipponbare). A) Plants after 10 days of emergence of the shoot and root in Ozawa's regeneration medium. B) *In vitro* regenerated rice seedling after one week in MS medium with Hygromycin, showing resistance to the selective agent; C) Regenerated seedling dead after one week in MS medium with Hygromycin, a clear sign of sensitivity to this selective agent.

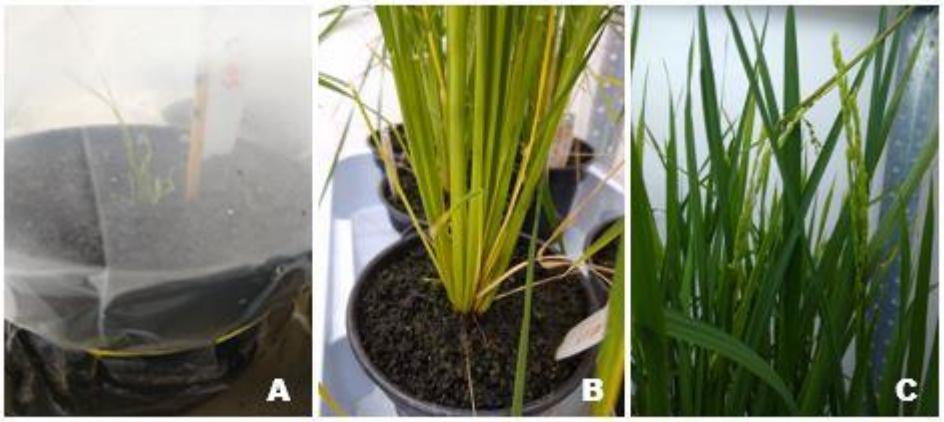

Figure 16. Rice plants (cv. Nipponbare) cultivated in soil. A) Seedling planted and bagged in soil. B) Two months old, highlighting the characteristic tillering of the species; C) Four months old in the initial stage of fruit development.

Soil cultivation was successful for most plants but not all. After the soil acclimatization phase, the plants were transferred to greenhouses subject to natural variations in temperature and light. To maximize seed yield, cultivation in 1L pots was prioritized, maintaining the soil soaked and free from competition with opportunistic plants. However, these cultivation conditions favored the proliferation of certain pests, particularly mites of the species *Oligonychus oryzae*, identified with the assistance of Dr. Liana Johann (UNIVATES). Pest control necessitated the application of acaricides, but the damage caused by the mites to the plants delayed their development and even led to the death of plants: Os02g1.1; Os02g5.1; Os02g8.1; Os02g10.1; Os05g1.2; Os05g4.1; Os05g4.2.

To confirm the presence of the transgene in the transformed plants, a PCR of gDNA samples was conducted (Fig. 17). For gene LOC_Os02g40900, primers Os02g40900_Ex1Antisense (forward) and ZmUbiqStartRv (reverse) were used, producing a 701 bp expected fragment, confirming the following plants: Os02g1.2, Os02g3.2, and Os02g4.1. For gene LOC_Os05g30140, primers Os05g30140_E5Fw (forward) and ZmUbiqStartRv generated the 322 bp expected fragment, confirming the Os05g3.1 plant. The WT sample and other plants being tested did not produce expected bands for the presence of the transgene and are probably not transgenic.

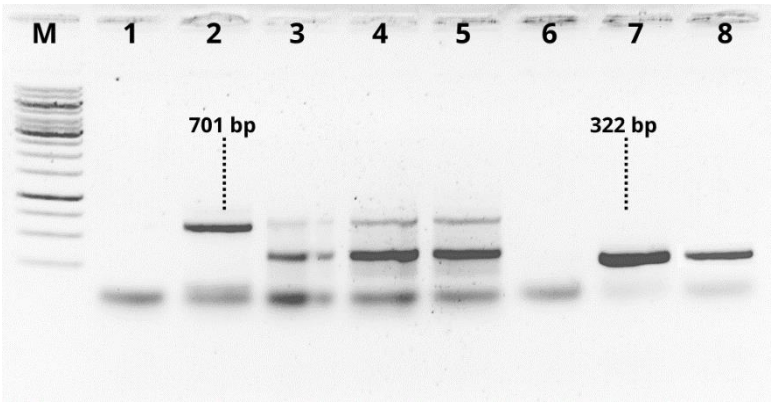

Figure 17. PCR to confirm transgene in transformed plants. Samples for LOC_Os02g40900 plants: lanes one to five. Samples for LOC_Os05g30140 plants: lanes six to eight; WT negative controls (lanes one and six, respectively); plasmid positive controls (lanes two and seven, respectively); confirmed transgenic LOC_Os02g40900 plants with expected band: Os02g1.2, Os02g3.2 and Os02g4.1 (lanes three to five, respectively); confirmed transgenic LOC_Os05g30140 plant Os05g3.1 with expected band (lane eight). M: GeneRuler™ 1 kb Plus DNA ladder (Thermo Scientific™).

To investigate if any of the transgenic plants had a deletion in the genomic region of the targeted genes, PCRs were conducted in transgenic plants. In the case of Os02g1.2, Os02g3.2, and Os02g4.1 plants, the primer pair Os02g40900_PromFw and Os02g40900_3UTRv could generate a more or over 1,131 bp fragment with the target deletion. In the absence of deletion, such as in the WT plants, the primers encompass a 3,585 bp region, which is virtually impossible to amplify via the standard PCR conditions used. None of the three transgenic plants presented a band, allowing the inference that none of the plants have the genomic deletion between PAM1 and PAM2 (Fig. 18). Similarly, for Os05g3.1, primers Os05g30140_PromFw and Os05g30140_I9Rv could result in a 1,244 bp fragment with the target deletion. The primers encompass a 4,451 bp region in WT plants, also indiscernible by PCR. Os05g3.1 did not present a detectable band, also allowing the inference that there is a genomic deletion between PAM1 and PAM2. (Fig. 19). The few transgenic plants obtained may explain why the respective genomic deletions did not occur, since it is already known that dual-targeting by CRISPR/Cas9 leads to efficient point mutagenesis, but only rare targeted deletions in the rice genome, somewhere around 2-4% in some cases (Srivastava *et al*., 2017; Pathak *et al*., 2019).

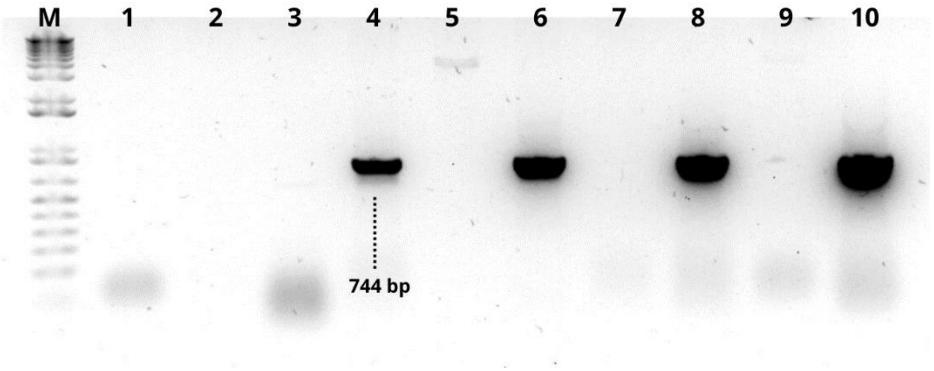

Figure 18. PCR to analyze genome editing in LOC_Os02g40900 in four transformed plants. Negative control with water as template (lane one); negative control with WT as template (lane two); negative and positive control for PAM1 (lanes three and four); Os02g1.2 (lanes five and six), Os02g3.2 (lanes seven and eight), Os02g4.1 (lanes nine and ten), in which the first lane of the sample pair denotes the CDS deletion, while the second lane denotes PAM1 editing. M: GeneRuler™ 1 kb Plus DNA ladder (Thermo Scientific™).

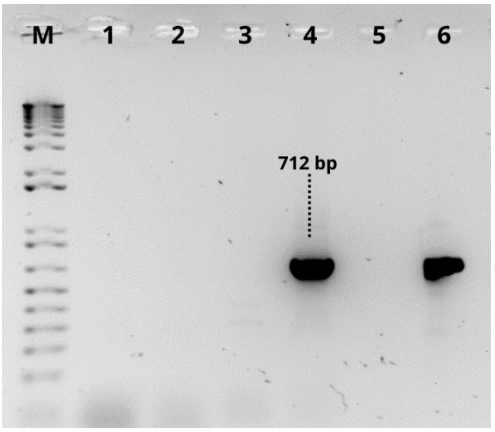

Figure 19. PCR for target deletion confirmation of LOC_Os05g30140 transformed plants. Negative and positive control for CDS deletion (lanes one and two, respectively); negative and positive control for PAM1 (lanes three and four); Os05g3.1 (lanes five and six), in which the lane five denotes the CDS deletion, while the lane six denotes PAM1 editing. M: GeneRuler™ 1 kb Plus DNA ladder (Thermo Scientific™).

Lastly, another reaction aimed to sequence the region around the first PAM of each target gene. For Os02g1.2, Os02g3.2, and Os02g4.1 plants, the primer pair Os02g40900_PromFw and Os02g40900_Ex1Rv produced a 744 bp fragment (Fig. 18). For Os05g3.1, primers Os05g30140_PromFw and Os05g30140_I2Rv generated a 712 bp fragment (Fig. 19). The PCR fragments from plants Os02g1.2, Os02g3.2, and Os02g4.1 were subjected to sequencing, revealing a sequence identical to the Nipponbare genomic sequence. Therefore, Os02g1.2, Os02g3.2, and Os02g4.1 plants possess the transgene,

but the sequenced material did not exhibit signs of gene editing. It is likely that genetic editing of LOC_02g40900 will occur as the plant develops, given that CRISPR activity tends to take place later in the development of T0 plants (discussed in Le *et al*., 2020).

On the other hand, the transgenic plant 05g3.1, containing the transgene targeted for editing LOC_Os05g30140, underwent editing in the targeted region (Fig. 20). This confirmation was possible by analyzing the sequencing of the genomic region around the first PAM using primers Os05g30140_PromFw and Os05g30140_I2Rv. All the confirmed transgenic plants (T0), identified through PCR, were maintained for seed production, facilitating future analyses with T1 and T2 generations, particularly in relation to their heat stress response.

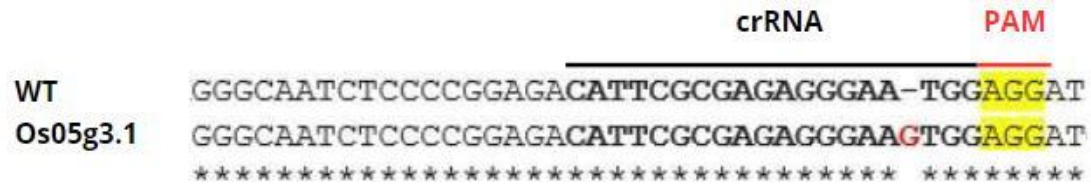

Figure 20. Target site mutation on Os05g3.1 T0. Yellow section indicates the PAM1 region, the 20 nt crRNA in bold black font, the guanine insertion in red, and the asterisk indicates equality between the two sequences.

VI. CONCLUSIONS

The strategies for obtaining the sgRNAs and transformation vectors were successful for both genes of interest. Due to the requirements of the selected promoter for sgRNA expression in rice, the tRNA methodology was necessary for the LOC_Os05g30140 gene, as opposed to the gene LOC_02g40900. This approach, although more challenging in experimental design with different series of PCR, allowed the establishment of two possible scenarios for sgRNA expression in the CRISPR/Cas9 gene editing strategy. The use of the Golden Gate Assembly® strategy for inserting each fragment of interest into a plant expression vector proved highly efficient, involving digestion and ligation of all fragments in a single reaction, which is faster than conventional cloning requiring separate digestions, gel purification, ligation, and transformation.

In this study, the plant transformation protocol required some adjustment and it eventually demonstrated efficiency. The initial steps of callus induction and co-cultivation with transformed *Agrobacterium* were highly effective and rapid, yielding a substantial volume of calluses with a low contamination rate. However, the regeneration step required most of the optimization, as the initial attempts were ineffective.

Although originally planned, T1 acquisition and heat stress assays were not carried out in this work due to unexpected plant cultivation conditions. The phenotype of selected T0 plants, including the number of tillers, height, and yield, could not be analyzed and compared with non-transformed plants under control conditions due to factors such as pests affecting plant development. Hopefully, another project carried out in the lab will analyze T1 plants in terms of phenotype, presence of transgene and gene editing. The identification of edited, transgene-free, homozygous knockout plants will be extremely useful in functional analysis.

Ultimately, the use of the CRISPR/Cas9 editing strategy for editing candidates for alternative splicing regulators under heat stress conditions proves to be a more efficient procedure compared to past methods, enabling a significant gain in scientific knowledge of how rice responds to temperature increases resulting from climate change in a short experimental period.

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