

CRISPR- CAS MEDIATED GENOME EDITING IN CROP PLANTS

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ABSTRACT:

Rice (*Oryza sativa* L.) is an important crop for the world population, but it is particularly vulnerable to salinity stress (high salt concentration), this stress is predominantly observed during the early stages and reproductive stages of the plant. This can impair plant growth causing water imbalance and buildup of toxin ions, which ultimately leads to yield loss. To overcome this, traditional breeding methods are used, but they are often very slow and imprecise due to the complexity involved in salt stress response traits. Although RNAi technology is used in knockdown of OsSIDP301 gene for reducing its expression and for limiting salt stress, Studies reveal that using CRISPR-Cas9 based knockout provides better tolerance and high yield as compared to knockdown using RNAi. Through CRISPR/Cas9 system the world is exposed to new possibilities in crop improvement. One promising target is OsSIDP301, A gene which codes for DUF1644-domain protein. Activated due to salt stress and ABA, reducing OsSIDP301 expression enhances salt tolerance and promotes increased grain size, which is likely due to modulation of sodium uptake and direct influence of stress-related genes such as OsHKT1;1 and ABI5. Furthermore, improvements are being achieved by knocking out the other regulatory genes such as OsRR22 and OsDST. These findings show us the potential of CRISPR-based genomic editing for improved rice varieties that are not only salt tolerant but high yielding too. This is an important step towards growing rice in salt-affected areas and sustainable agriculture.

KEYWORDS: CRISPR/CAS9, Salinity Tolerance, gRNA, OsSIDP301, RNAi, Knockout

Introduction

Rice (*Oryza sativa* L.) is one of the most significant cereal crops and the main source of calories for more than half of the world's population (Khush, 2013). It is also regarded as a model plant for cereal genomics; however, abiotic pressures can seriously harm it (Lafitte *et al.* 2004). Abiotic stress limits plant yield and product quality by interfering with normal growth and development (Boyer, 1982; Pandey *et al.*, 2017). Rice is a species that was first cultivated in freshwater marshes and swamps (Dionisio-Sese and Tobita 1998; Kumar *et al.* 2013). Consequently, it is considered a particularly salt-sensitive crop and is extremely vulnerable to salt stress during the reproductive and young seedling phases (Reddy *et al.* 2017). Salinity is a significant environmental factor that restricts crop yield. When the electrical conductivity of saline soils exceeds 4 dS/m (4 dS/m & 40mM NaCl; Qadir *et al.* 2000), many crops cannot grow. Owing to their salt sensitivity, the yields of several rice cultivars can be decreased by as much as 50% when exposed to 50 mM NaCl (Radanielson *et al.* 2018).

Plant growth is influenced by salinity in two stages. First, by lowering the water potential, which is comparable to the effect of osmotic stress. In response to this stress, plants translocate solutes to their roots, close their stomata, and slow down the growth of their shoots. Second, a slower ionic phase ensues, causing harmful salt levels to build up in the leaves, hastening senescence, and decreasing the absorption of different nutrients. Approximately 30% of rice-growing regions are negatively impacted by soil salinity (Negrão *et al.* 2011; Fang *et al.* 2023; Khan *et al.* 2024). Consequently, the development of cultivars that can withstand salt is crucial for rice breeding initiatives (Padmavathi *et al.* 2023; Tiwari *et al.* 2024).

Plants undergo morphological, physiological, biochemical, and molecular alterations in response to abiotic stress (Wang *et al.*, 2016). Additionally, it may take years to breed stress-tolerant cultivars in the old-fashioned manner, which means that agricultural researchers may take longer to respond to the changing needs of growers, farming communities, and industries (Manavalan *et al.*, 2009). In recent decades, the development of crop plants that can withstand abiotic stress has been transformed by genome-editing technology, which is regarded as an important tool. In a variety of organisms, including plants, the most effective genome editing method has been the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (CRISPR/Cas9) system (Chen *et al.*, 2019). By targeting multiple stress-sensitive genes simultaneously in a high-yielding but sensitive cultivar, CRISPR technology holds great promise for creating genome-edited crop plants that are tolerant to multiple stresses. Additionally, tolerance genes can be overexpressed using CRISPR-

mediated gene activation (Zafar *et al.*, 2020). The groundbreaking gene-editing technique CRISPR-Cas9 has revolutionized genetic engineering by offering an accurate and effective method for making specific changes in an organism's DNA. The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) proteins that bacteria use to defend against viral infections serve as the basis for this technology (Doudna and Charpentier, 2014). In plants, negative regulatory genes frequently serve as molecular "brakes" that reduce the intensity of stress reactions. In an effort to increase plant resilience by lifting repression overprotective pathways, recent studies have used CRISPR/Cas systems to knock out these genes in plants.

Despite reports that OsSIDP366 and OsSIDP361 are implicated in abiotic stress (Guo *et al.*, 2016; Li *et al.*, 2016), the coordinated regulatory network between salt tolerance and grain size remains unknown. Gene overexpression (OE), gene silencing (RNA interference; RNAi), and gene knock-out were developed in plants in the study by Ge *et al.* (2022) to investigate the function of OsSIDP301, which encodes a DUF1644 protein. The stress-induced DUF1644 protein, OsSIDP301, was triggered by both salt stress and abscisic acid (ABA). In contrast, plants with OsSIDP301 RNA interference (RNAi) showed salt tolerance and larger grain sizes, whereas plants with overexpression of OsSIDP301 (OE) showed salt hypersensitivity and smaller grain sizes (Ge *et al.*, 2022). Salt-response and ABA signaling pathway genes were modulated by OsSIDP301 to determine salt stress tolerance. According to study, OsSIDP301 may control grain size by affecting spikelet hull cell expansion. Additionally, OsSIDP301 interacts with OsBUL1 COMPLEX1 (OsBC1), which positively controls rice grain size. OsSIDP301 is a negative regulator of salt stress and grain size (Ge *et al.*, 2022), and suppressing its expression is a viable tactic to increase rice productivity and tolerance to salt stress.

Knockdown vs Knockout of OsSIDP301

Knockout refers to the complete disruption or deletion of a gene, typically achieved through targeted mutations like CRISPR/Cas9, leading to the absence of a functional protein. In the study conducted by (Ge *et al.*, 2022) demonstrates that RNA interference (knockdown) reduces OsSIDP301 expression and produces favorable results. Although the study of (Ge *et al.*, 2022) does not explicitly mention a knockout, it is logical to infer that a full knockout, which entirely abolishes OsSIDP301 function, might result in similar or even more pronounced beneficial effects (such as enhanced salt tolerance and larger grain size) because the gene's negative regulatory role would be eliminated. OsSIDP301 (stress-induced DUF1644 protein) is a rice gene activated by salt stress and abscisic acid (ABA). Overexpressing OsSIDP301 leads to increased salt sensitivity and smaller grain size. Conversely, reducing OsSIDP301 expression through RNA interference (RNAi) improves salt tolerance and increases grain size. This suggests that OsSIDP301 functions as a negative regulator of both salt stress tolerance and grain size, implying that its normal role suppresses these traits (Ge *et al.*, 2022). Given OsSIDP301's role as a negative regulator, creating a knockout mutant could be a promising strategy for enhancing both salt stress tolerance and grain yield in rice. This approach might be more effective than knockdown if the complete loss of function does not result in unintended negative effects. The study of (Guo, *et al.*, 2018) reveals that reduced expression (knockdown) of GSN1 (OsMKP1) leads to larger but fewer grains, while increased expression results in more but smaller grains. Complete suppression (knockout) of related mitogen-activated protein kinase genes (OsMPK6, OsMKK4, and OsMKKK10) led to denser panicles and smaller grains, indicating that knockout can have distinct and potentially more severe effects than knockdown. The findings suggest that knocking out a negative regulator, such as OsSIDP301, may produce stronger or different phenotypes compared to knockdown, potentially affecting both salt stress response and grain size more dramatically (Guo, *et al.*, 2018). Knocking out a gene that negatively regulates grain size and salt stress tolerance, as demonstrated for GW5L in rice, can result in increased grain size and improved salt stress resistance in the crop. The study conducted by (Tian, *et al.*, 2019) shows that knockout mutants of negative regulators, like GW5L, are effective in enhancing both grain yield and salt stress tolerance, suggesting that knockout is a viable alternative to knockdown for similar genes, such as OsSIDP301.

CRISPR/Cas9-based genome editing method: A mechanistic review.

The adaptive immunological mechanism that protects bacteria and archaea from the invasion of foreign plasmids or viral DNA is the foundation of the CRISPR/Cas system (Marraffini and Sontheimer, 2010). The CRISPR/Cas9 system consists of two components: CRISPR-associated protein 9 (Cas9) and one guide RNA (sgRNA) (Cong *et al.*, 2013). Two different RNAs that are synthesized into sgRNA and necessary for CRISPR action are protospacer-matching CRISPR RNA (crRNA) and transactivating crRNA.

Target genomic region is attached to the sgRNA by 20 nucleotides at its 5' end as part of the sgRNA/Cas9 combination. The protospacer adjacent motif (PAM; NGG for SpCas9 from *Streptococcus pyogenes*) is a short, conserved DNA sequence downstream of the cleavage site that changes in size depending on the bacterial species. This target site must be adjacent to the PAM. The large (1368 amino acid) multi-domain DNA endonuclease SpCas9 cleaves target DNA in the genome, resulting in a blunt-ended double-strand break (DSB). Lastly, DSB is fixed by the host cellular machinery (Mei *et al.*, 2016).

The two techniques used to fix DSBs brought on by the Cas-9 protein are homology-directed repair (HDR) and non-homologous end joining (NHEJ) (Liu *et al.*, 2018). Using a homologous DNA template, homology-directed repair is very accurate. HDR needs several donor DNA templates with a target DNA sequence and is most active in the late S and G2 phases of the cell cycle.

A donor DNA template with sequence homology is introduced at the expected DSB site to perform selective gene replacement or insertion (Liu *et al.*, 2018; Yang *et al.*, 2020). In order to speed up DSB repair, non-homologous end-joining links DNA fragments utilizing an enzymatic method instead of exogenous homologous DNA.

In terms of precision, homology-directed repair, which is achieved through homologous recombination (HR) is usually the most effective technique for gene editing. It can incorporate a sequence that is provided by the donor template, enabling editing without errors. HDR-mediated Cas9 may insert resistance (R) genes, making it one of the most popular genomes editing techniques for biological applications such as knock-in or precision mutagenesis. This technique improves plants' capacity to respond to stress. The more accurate HDR repair method uses homologous donor DNA to fix damaged DNA, in contrast to the error-prone NHEJ technique. While HDR can be used to generate precise point mutations or insert specified sequences by homologous recombination, NHEJ introduces indels (Jain 2015).

The production and analysis of CRISPR-altered crop plants has been facilitated by several technical advancements in recent years. A major technological obstacle to the application of CRISPR in plants is its low intrinsic HDR efficiency, which precludes the realization of many of the proposed applications, including extensive chromosomal deletions and gene substitutions. There are disadvantages to the various HDR-improving techniques that have been explored.

Through template-mediated and HDR of DSB, the target *als* gene in sugarcane is precisely replaced, giving these CRISPR-edited plants herbicide tolerance. It is possible to stack or pyramid many genes involved in a regulatory network or stress response pathway using HDR-mediated gene targeting. Since tandem repeated sequences near DSBs increase HDR efficiency, a tandem repeat-HDR (TR-HDR) approach to gene targeting was later developed. In rice, the editing efficiency varied from 3.4 to 11.4%, and in-locus tags were successfully inserted using TR-HDR (Ali *et al.*, 2020). This technique, known as RNA-guided genome editing (RGE), uses the bacterial type II cluster regularly interspaced short palindromic repeats (CRISPR)–associated nuclease (Cas) to modify the genomes of many organisms, including plants and crops, in a simple and flexible way.

The *Streptococcus pyogenes* CRISPR-Cas9 system is the basis for RGE, in which a short single guide RNA (gRNA or sgRNA) instructs the Cas9 nuclease to generate double-strand breaks (DSB) at particular sites of chromosomal DNA. Because non-homologous end joining repair (NHEJ) is prone to errors, this results in mutations at the DSB.

Cas9-gRNA can recognize the targeted DNA based on the complementarity between a gRNA spacer (about 20 nt long leading sequence of gRNA) and its target DNA, which appears before a protospacer-adjacent motif (PAM, Figure 2). Using the CRISPR-Cas9 system and *Agrobacterium*-mediated transformation, we describe the main procedures for plant RGE. The process, which includes gRNA design, Cas9-gRNA plasmid synthesis, and mutation detection (genotyping) for rice RGE, may be adjusted for other plant species. RNA interference (RNAi)-mediated knockdown was once the most widely used technique for decreasing the number of cells expressing a particular gene product. But usually, RNA interference doesn't halt a gene entirely. Artificial nuclease-mediated gene modification, on the other hand, alters genetic coding and typically results in a "knockout," or complete loss of gene function.

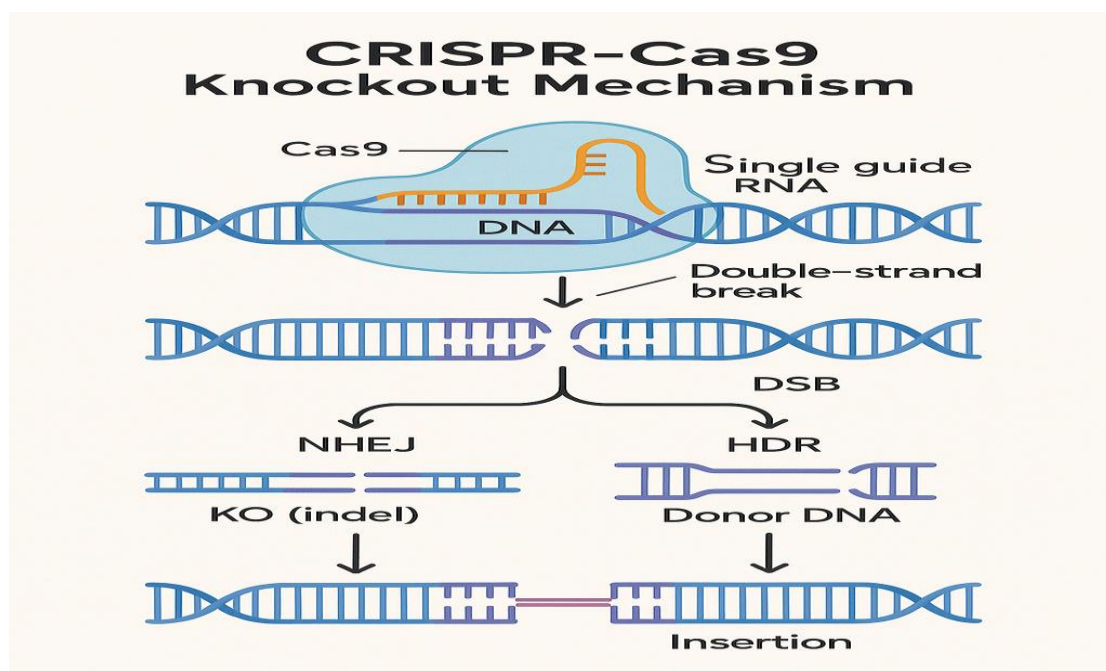


Figure1: This diagram illustrates the knockout of negatively regulating genes and knock in of positively regulating genes by using CRISPR-Cas9 technology

OsSIDP301: A Negative Regulator of Salt Tolerance and Grain Size

The Stress Induced DUF1644 Protein 301 (OsSIDP301) is a negative regulator for both salt stress and grain size in rice (Ge *et al.* 2022) and positively regulates abscisic acid signaling

OsSIDP301 regulates the tolerance of rice to salt stress through the abscisic acid (ABA) signaling pathway and affects grain size by influencing cell expansion in spikelet hulls (Ge *et al.* 2022)

According to Mistry *et al.* (2021), 24% (4795/19,632) of the protein families currently listed in the Protein Family (Pfam) database (version 35.0) are uncharacterized functional proteins (UPFs) and proteins that contain domains of unknown function (DUFs). These protein families are collectively referred to as the DUF family. Protein domains with relatively conserved amino acid sequences and unknown activities are known as DUFs (Bateman *et al.* 2010). However, the precise molecular processes of most members of this family are unknown. Over the last 20 years, significantly more DUF genes have been discovered in the plant kingdom. Domains with an unclear function 1644 is a large protein group (DUF1644) in the DUF family (Bateman *et al.*, 2010). Using a reverse genetics approach, OsSIDP301, a member of the DUF1644 protein family, was identified as a negative regulator of salt stress tolerance and seed development in rice, functioning through modulation of ABA signaling and interaction with OsBC1, a positive regulator of grain size (Ge *et al.*, 2022; Luo *et al.*, 2024)

Rice cultivation is severely hampered by salt stress, which affects biomass, growth, and total output (Xu *et al.*, 2024). The function of OsSIDP301 in regulating rice reactions to salinity has been investigated in recent studies. It has been demonstrated that OsSIDP301 knockdown improves salt tolerance in rice by lowering sodium (Na) buildup (Ge *et al.*, 2022). Also, the knockdown of OsSIDP301 reduces the ROS levels in rice under salt stress (Ge *et al.*, 2022). To ascertain whether osmotic stress was the cause of the decreased growth and biomass seen in OsSIDP301-overexpressing (OsSIDP301OE) plants, seedlings were subjected to different mannitol concentrations (0, 120, and 150 mM). Ge *et al.* (2022) found no significant differences between OsSIDP301OE and wild-type (WT) plants, suggesting that ion toxicity, rather than osmotic stress, contributed to their increased sensitivity to salinity.

Quantitative analysis of Na⁺ content in the leaves of four-week-old rice seedlings subjected to NaCl treatment revealed significant differences among genotypes. Notably, OsSIDP301 overexpression (OE) lines accumulated substantially higher levels of Na⁺ compared to wild-type (WT) plants, whereas RNA interference (RNAi) lines exhibited reduced Na⁺ accumulation, suggesting that OsSIDP301 knockdown enhances salt tolerance by limiting Na⁺ uptake (Ge *et al.*, as cited in Srivastava *et al.*, 2024).

Further transcriptomic profiling uncovered differential regulation of key salt-responsive genes. The ABA signaling repressor ABI5 was significantly upregulated in OsSIDP301OE plants, while positive regulators of salt tolerance—including OsHKT1;1 (Wang *et al.*, 2015), OsHAK21 (Shen *et al.*, 2015), OsHAK22 (He *et al.*, 2019), OsP5CS (Zhang *et al.*, 1995), and OsNCED1 (Liu, 2017) were markedly downregulated, reinforcing the role of OsSIDP301 as a negative regulator of salt stress response in rice (Srivastava *et al.*, 2024)

In contrast to overexpression lines, OsSIDP301RNAi plants exhibited reduced Na⁺ accumulation and enhanced salt tolerance, confirming that RNAi-mediated knockdown, which partially suppresses gene expression, can effectively modulate stress responses in rice (Ge *et al.*, 2022). These findings underscore the regulatory role of OsSIDP301 in salt stress adaptation, where its suppression leads to improved physiological performance under saline conditions. Collectively, the results by Ge *et al.* (2022) suggest that OsSIDP301 modulates the expression of key salt-responsive genes and influences Na⁺ homeostasis, thereby altering the plant's sensitivity to salt stress. This highlights the potential of targeted OsSIDP301 regulation as a strategy to enhance salinity tolerance, contributing to more sustainable rice cultivation practices. To further elucidate OsSIDP301's role in plant development, Ge *et al.* identified OsBC1, a basic helix–loop–helix (HLH) transcription factor, as a direct interactor of OsSIDP301. Notably, silencing of OsBC1 led to reduced grain size, indicating its positive role in grain development. These findings demonstrate that OsSIDP301 simultaneously regulates salt stress tolerance and grain size, offering a dual-target opportunity for genetic improvement in rice.

Knockout of OsSIDP301

To produce knockout mutants, two guide RNAs (gRNAs) were designed to target a particular 20-nucleotide region within the DUF1644-encoding exon of OsSIDP301, which is situated directly upstream of an NGG protospacer adjacent motif (PAM), in order to guarantee that Cas9-induced indels would cause frameshifts and the loss of the protein's functionality (Ge *et al.*, 2022). The pH-Ubi-Cas9 binary vector, in which *Streptococcus pyogenes* Cas9 is driven by the maize ubiquitin promoter for robust expression in monocots, was created by cloning each gRNA under the control of the rice U6 promoter (Chen *et al.*, 2009). Using well-established transformation techniques, these constructs were inserted into the japonica rice cultivar TP309 by *Agrobacterium tumefaciens* strain EHA105 (Ge *et al.*, 2022; Shan *et al.*, 2013). Cas9/gRNA ribonucleoproteins caused double-strand breaks at the target exon site in the transformed calli. These breaks were fixed by the non-homologous end-joining (NHEJ) pathway, resulting in minor insertions or deletions (Ran *et al.*, 2013). Two distinct homozygous alleles (cas9-2 and cas9-3) with frameshifts close to the N-terminal domain that introduced premature stop codons downstream of DUF1644 were found in T₀ generation plants after PCR and Sanger sequencing screening (Ge *et al.*, 2022). Following that, these T₁ homozygotes were chosen as null mutants for subsequent confirmation of the grain size modulation and salt stress response (Ge *et al.*, 2022).

Knockout of other negatively regulating genes

Transcription factors (TFs) and other regulatory elements may govern the defensive mechanism [Wang, *et al.*, 2016]. TFs can either positively or negatively control gene expression by binding to cis-acting regions in the promoters of target genes (Latchman *et al.*, 1993). One of the main ways in which plants maintain growth, development, and defense against a variety of environmental challenges is through the regulation of TFs (Chen *et al.*, 2004). One of the biggest TFs, the plant basic-helix–loop–helix (bHLH), is made up of 25 subfamilies that protect plants from abiotic stressors such as salt stress (Li, X.; *et al.* 2006). Of these, the OsRR22 gene produces a transcription factor, a 696-amino acid B-type response regulator, implicated in metabolism and cytokinin signal transmission; its deficiency has been shown to dramatically increase salt tolerance (Takagi *et al.* 2015). Many genes (OsPDS, OsERF922, OsHAK1, Badh2, and TMS5) have been knocked out in rice using CRISPR/Cas9 technology, yielding the desired phenotypes (Zhang *et al.* 2014; Wang *et al.* 2016; Zhou *et al.* 2016; Nieves-Cordones *et al.* 2017; Shao *et al.* 2017). This technique offers a novel approach to rice breeding. OsRR22 is the first transcription factor to reveal that CRISPR/Cas9-targeted mutagenesis to improves salinity tolerance.

Besides allele insertion-deletion and knockout, the CRISPR editing technique also produces tolerant plants by altering genomic features through transcript activation, epigenetic alteration through chromatin unwinding, and promoter region switching with HDR (LiX *et al.*, 2022). The distinct expression profiles of the rice response regulators OsRR9 and OsRR10 across developmental stages, tissues, and environmental conditions suggest their functional divergence in stress adaptation. These type-A response regulators are transcriptionally active during various growth phases, with particularly elevated expression observed during the booting stage (Wang *et al.*, 2019). Both genes are expressed in leaf tissues throughout development, indicating a sustained role in vegetative signaling (Saha *et al.*, 2024). Notably, transcript abundance is significantly higher in shoots compared to roots or panicles, implying a tissue-specific regulatory function potentially linked to shoot development and environmental responsiveness (Wang *et al.*, 2019). This spatial and temporal variation in expression supports the hypothesis that OsRR9 and OsRR10 contribute differentially to cytokinin-mediated signaling pathways under abiotic stress conditions (Saha *et al.*, 2024).

The results from the study conducted by (Wang *et al.*, 2019) suggest that OsRR9 and OsRR10 contribute to various aspects of rice stress adaptation, with OsRR10 responding differentially to osmotic and salt stress and OsRR9 contributing to heat tolerance. By using targeted genetic techniques, it may be possible to improve rice tolerance to stress by better understanding the regulatory mechanisms.

The OsDSG1 gene, which plays a role in the ubiquitination pathway and regulation of biochemical processes in rice, was precisely targeted using CRISPR/Cas9 technology in a local indica cultivar to generate heritable mutations (Ly *et al.*, 2024). These CRISPR/Cas9-induced OsDSG1 mutant lines exhibited enhanced salt tolerance at both germination and seedling stages, as demonstrated by increased plant height, root length, total fresh weight, chlorophyll content, and relative water content under salt stress conditions (Ly *et al.*, 2024). Additionally, the mutants accumulated lower levels of proline and malondialdehyde (MDA) compared to wild-type plants under stress, while showing no significant differences in growth or seed germination under normal conditions (Ly *et al.*, 2024). These findings suggest that OsDSG1 contributes to salt stress resistance and may serve as a promising target for improving stress resilience in local rice varieties. Similarly, the *dst* (N69D) mutation, originally identified in a Japanese rice cultivar, was phenocopied in the indica cultivar MTU1010 using EMS mutagenesis, resulting in *dstD184–305* mutants with wider leaves and reduced stomatal density under dehydration stress—traits that enhance water retention (Kumar *et al.*, 2020). This reduction in stomatal density was linked to the downregulation of key stomatal development genes such as SPCH1, MUTE, and ICE1 (Kumar *et al.*, 2020). The Cas9-free *dstD184–305* mutants also demonstrated strong salt tolerance and moderate osmotic stress tolerance during the seedling stage, indicating their potential for improving drought and salt resilience in indica rice (Kumar *et al.*, 2020). The OsDST gene encodes a zinc-finger transcription factor, and its function is critically dependent on the conserved N69 residue within the zinc finger motif (Huang *et al.*, 2009). Mutations such as N69D and A162T alter the amino acid sequence of the DST protein, with transgenic complementation studies confirming that the N69D substitution is primarily responsible for the *dst* mutant phenotype (Kumar *et al.*, 2020; Huang *et al.*, 2009). By altering H₂O₂ homeostasis, this mutation increased stomatal closure, decreased stomatal density, and widened the leaf. The N69D mutation caused the DST protein to lose its function, which improved rice resistance to salt and drought (Huang *et al.* 2009).

OsDST	LOC_Os03g57240	Agrobacterium Mediated	(Santosh Kumar <i>et al.</i> , 2020)
OsRAV2	LOC_Os01g04800	Agrobacterium Mediated	(Duan <i>et al.</i> ,2016)
OsRR22	KF892986	Agrobacterium Mediated	(Zhang <i>et al.</i> ,2019)
OsNAC45	KT957809	Agrobacterium Mediated	(Zhang <i>et al.</i> ,2020)

OsBBS1	LOC_Os03g24930	Agrobacterium Mediated	(Zeng <i>et al.</i> , 2018)
OsAGO2	LOC4336991	Agrobacterium Mediated	(Yin <i>et al.</i> , 2020)
OsVDE	LOC_Os04g31040	Agrobacterium Mediated	(Wang <i>et al.</i> , 2003)
OsNAC041	LOC_Os03g013300	Agrobacterium Mediated	(Bo <i>et al.</i> , 2019)
OsSAPK2	LOC_Os07g42940	Agrobacterium Mediated	(Lou <i>et al.</i> , 2017)
OsPQT3	LOC_Os10g29560.1	Agrobacterium Mediated	(Alfatih <i>et al.</i> , 2020)
OsPIL14	LOC_Os07g05010	Agrobacterium Mediated	(Mo <i>et al.</i> , 2020)
OsBGE3	LOC_Os01g48800	Agrobacterium Mediated	(Yin <i>et al.</i> , 2017)
OsSPL10	LOC_Os06g44860	Agrobacterium Mediated	(Lan <i>et al.</i> , 2019)
OsDOF15	LOC_Os03g55610	Agrobacterium Mediated	(Qin <i>et al.</i> , 2019)
OsFLN2	AP014960	Agrobacterium Mediated	(Chen <i>et al.</i> , 2020)

The regulatory role of the GT-1 element in rice promoted CRISPR/Cas9-mediated editing of OsRAV2 gene expression, which demonstrated resistance to salt stress (Duan *et al.*, 2016). Rice was resistant to salt stress when CRISPR mutants with loss of function of SnRK2 and osmotic stress/ABA-activated protein kinases SAPK-1 and SAPK-2 genes were present (Lou *et al.*, 2017). In a number of other studies, CRISPR-mutants were created in rice to produce plants that can withstand salt stress by knocking out OsDST (Santosh Kumar *et al.*, 2020), OsNAC45 (Zhang *et al.*, 2020), AGO2 (ARGONAUTE2) (Yin *et al.*, 2020), Rice type-B response regulator (OsRR22) (Zhang *et al.*, 2019), and OsBBS1 (bilateral blade senescence1) (Zeng *et al.*, 2018).

CRISPR/Cas9-generated wheat plant TaHAG1 gene knockout mutants shown improved salt tolerance (Zheng *et al.*, 2021). In order to investigate the function of the OsbHLH024 gene under conditions of salt stress, Alam *et al.* (2022) created rice mutant plants with altered genes. When exposed to salt stress, the osbhlh024 mutants' total chlorophyll content and shoot biomass increased considerably, indicating the deletion of one nucleotide base (Alam *et al.*, 2022). Growth, stress reactions, and ABA biosynthesis. The functional advantages of OsVDE in salt tolerance in rice have been confirmed.

The transcription factor Through limiting ethylene production, OsDOF15 controls cell proliferation in the root meristem, hence favorably regulating primary root elongation. Primary root extension and root meristem cell proliferation were hampered by OsDOF15 loss of function (Qin *et al.*, 2019). The DELLA protein SLENDER RICE1 (SLR1) and OsPQT3 are two examples of negative regulators for salt stress tolerance that can be edited. Alfatih *et al.* (2020) found that OsPQT3 knockout mutants in rice showed improved resistance to oxidative and salt stress due to increased expression of OsGPX1, OsAPX1, and OsSOD1 under salt stress, while SLR1 loss facilitated mesocotyl and root growth, particularly in the dark and under salt stress (Mo *et al.*, 2020).

Experimental Validation of OsSIDP301 Function

To elucidate the molecular interactions and expression patterns of OsSIDP301, Ge *et al.* (2022) employed a combination of yeast two-hybrid (Y2H) screening, bimolecular fluorescence complementation (BiFC), and GUS reporter assays. In the Y2H assay, OsSIDP301 was cloned into the pGBKT7 vector and used as bait to screen a rice seedling cDNA library, identifying OsBC1—a bHLH transcription factor involved in grain size regulation—as a direct interactor. BiFC analysis further confirmed nuclear interactions between OsSIDP301 and OsBC1, as well as OsSIDP301 homodimerization, through strong YFP fluorescence in *Nicotiana benthamiana* leaf cells. To assess spatial expression, a 988-bp OsSIDP301 promoter fragment was fused to the GUS reporter gene and introduced into rice using the pCXGUS-P vector [Chen *et al.*, 2009], revealing strong GUS activity in panicles and spikelet's, indicative of developmental regulation [Ge *et al.*, 2022].

CONCLUSION:

Rice (*Oryza sativa* L.) is a vital staple food for much of the world, but its productivity is greatly affected by abiotic stresses, especially soil salinity. With the increasing salinization of agricultural lands, there is an urgent need to develop rice varieties that can tolerate salt stress. Traditional breeding methods have had limited success due to the complex nature of salt stress responses. However, modern molecular tools, especially the

CRISPR/Cas9 gene-editing system, have opened new possibilities for improving stress resistance in rice by precisely targeting specific genes. One such gene, *OsSIDP301* function loss modifies ion homeostasis, oxidative stress responses, and cell-expansion pathways, resulting in enhanced salt tolerance and larger grain size. Two guide RNAs that target the *OsSIDP301* coding area were cloned into the rice Cas9 binary vector (pH-Ubi-Cas9) and then inserted into the TP309 background using *Agrobacterium* manipulation. Following the identification of independent T0 lines with minor deletions in the exon that codes for DUF1644 using PCR and sequencing, two homozygous frame-shift alleles (*cas9-2* and *cas9-3*) were advanced for phenotypic examination Ge., *et al* 2022). *OsSIDP301* gene expression increases in response to salt stress and the hormone abscisic acid (ABA). Overexpression of *OsSIDP301* leads to smaller grains and higher salt sensitivity. On the other hand, reducing its expression using RNA interference (RNAi) improves salt tolerance and increases grain size. The gene appears to enhance ABA signalling while suppressing stress-tolerance genes like *OsHKT1;1*, *OsHAK21*, *OsHAK22*, *OsNCED1*, and *OsP5CS*, and activates *OsABI5*, a salt stress inhibitor. Knockdown of *OsSIDP301* reduces sodium and reactive oxygen species accumulation, improving plant health under saline conditions. CRISPR/Cas9 allows for efficient gene knockouts, making it an ideal method to eliminate negative regulators like *OsSIDP301*. While a full knockout wasn't performed in (Ge *et al.* 2022), their findings suggest that such an approach could further boost salt resistance and yield. Similar CRISPR-based knockouts of *GW5L*, *OsRR22*, *OsDST*, and *OsPQT3* have already improved tolerance to salt and drought without reducing crop performance. Additionally, *OsSIDP301* interacts with the bHLH transcription factor *OsBC1*, which promotes grain size, suggesting a dual role in managing both stress and yield.

Further evidence from yeast two-hybrid and GUS assays supports that *OsSIDP301* functions in the nucleus and plays a central role in gene regulation. These results highlight its importance in controlling ion balance, ABA response, and stress-related transporters. Given this, *OsSIDP301* is a strong candidate for targeted genome editing to improve rice performance in saline soils. CRISPR-based editing of this gene, validated through transcriptomic and phenotypic analyses, offers a promising direction for breeding programs aimed at developing salt-resilient rice varieties. CRISPR/Cas9 is a cutting-edge tool that enables scientists to make specific changes to plant DNA. It is now widely used to enhance crop tolerance to stressors like salinity, drought, and heat. In rice, targeting *OsSIDP301* through knockdown or knockout approaches helps plants grow better under salt stress while also producing larger grains. CRISPR works by using a guide RNA and the Cas9 protein to cut DNA at specific sites, either deactivating harmful genes or enhancing beneficial ones. This technology is faster and more accurate than traditional breeding and has shown great promise in creating crops that can thrive in harsh environments, helping secure food production for the future.

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