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CRISPR-CAS MEDIATED FUNCTIONAL CHARACTERIZATION OF GENES GOVERNING ROOT ARCHITECTURE, WATER USE EFFICIENCY, AND DROUGHT TOLERANCE IN RICE

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Abstract

Rice (Oryza sativa L.) is the staple food for over half the global population, yet its semi-aquatic evolutionary origin renders it highly vulnerable to drought, the most devastating abiotic constraint on productivity. Climate change, characterized by erratic precipitation, rising temperatures, and prolonged drought events, threatens the 25% yield increase required to feed 8.5 billion people by 2030. This review elucidates CRISPR-Cas-mediated functional characterization of genes governing root system architecture (RSA), water use efficiency (WUE), and drought tolerance in rice. We examine the evolution of CRISPR-Cas toolkits from canonical SpCas9 and high-fidelity variants to Cas12a, multiplex tRNA/U3 arrays, and transgene-free ribonucleoprotein delivery that enable precise dissection of polygenic stress traits and functionally redundant gene families. Functional analyses of RSA regulators, including OsPIN1b, OsRPK1, DRO1, OsWOX11, OsDRP1C, OsOPR5, OsDUF1223, and OsNAC6, reveal their roles in auxin transport, gravitropism, crown root initiation, and deep rooting, collectively enhancing subsoil water capture. We further discuss stomatal patterning genes (OsEPFL9, OsEPF1, OsDST, OsDIRH2) that modulate stomatal density and ABA sensitivity to optimize intrinsic WUE, and phytohormonal

networks (OsPYL family, OsNAC016, OsNAC14, OsERA1) governing ABA signaling and BR-ABA crosstalk. Finally, we address agronomic trade-offs between drought adaptation and yield, and highlight multi-trait pyramiding, promoter editing, base/prime editing, and transgene-free delivery as strategies for developing climate-resilient, high-yielding rice cultivars.

INTRODUCTION

Global agricultural productivity faces compounding challenges driven by climate change, manifested through unpredictable precipitation patterns, escalating ambient temperatures, and prolonged severe drought events. Cultivated rice (*Oryza sativa* L.) serves as the primary staple food for more than half of the global human population, with over 90% of global production concentrated across Asian agricultural ecosystems (Wang et al., 2026). Projections indicate that the global human population will expand to 8.5 billion by 2030, requiring an estimated 25% increase in rice grain yield under increasingly adverse climatic conditions. Rice is intrinsically susceptible to water deficits due to its evolutionary adaptation to semi-aquatic habitats, rendering drought the single most devastating abiotic constraint limiting productivity across both *indica* and *japonica* production systems (Khan et al., 2021).

To sustain physiological function under water limitation, rice plants deploy adaptive survival strategies broadly categorized into drought avoidance, drought tolerance, and drought recovery mechanisms. Drought avoidance relies on structural and physiological adaptations that minimize transpirational water loss or optimize subterranean moisture capture (Sandeep & Godi, 2023). Central to this strategy are modifications in Root System Architecture (RSA) including primary root length, crown root density, lateral root branching, and root growth angle and the precise regulation of leaf stomatal dynamics to optimize Water Use Efficiency (WUE) (Parmar et al., 2025). Conversely, drought tolerance involves cellular metabolic adjustments, such as osmotic balance maintenance, reactive oxygen species (ROS) detoxification, and hormone-mediated transcriptional reprogramming driven primarily by abscisic acid (ABA), auxins, brassinosteroids (BRs), and jasmonates (JA) (Ogata et al., 2020).

Deciphering the molecular pathways governing these complex quantitative traits historically relied on forward genetics, physical/chemical mutagenesis (e.g., ethyl methanesulfonate [EMS]), and quantitative trait locus (QTL) mapping. Although these classic approaches led to the discovery of major loci such as *DEEPER ROOTING 1* (*DRO1*), they were often constrained by genetic linkage drag, labor-intensive gene identification, and functional redundancy within multigene families (Shelake et al., 2022). The emergence of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated (Cas) nucleases has transformed plant functional genomics. CRISPR-Cas technology enables precise, targeted single and multiplex gene disruptions, base edits, and transcriptional modulations, allowing researchers to uncouple complex genetic networks in elite rice germplasm (Chatterjee et al., 2025). This review provides a comprehensive synthesis of CRISPR-Cas-mediated functional characterization of candidate genes regulating RSA, stomatal-mediated WUE, and drought tolerance networks in rice, emphasizing physiological mechanisms, regulatory crosstalk, and translational applications for climate-resilient crop breeding (Usman et al., 2020).

To visualize the holistic pipeline from initial gene discovery to the generation of climate-resilient cultivars, the overarching workflow is systematically outlined in **Figure 1**. This schematic illustrates the four critical phases of the technology: abiotic stress-responsive gene discovery, CRISPR-Cas ribonucleoprotein assembly, double-stranded DNA break repair via non-homologous end joining (NHEJ) or homology-directed repair (HDR) pathways, and the subsequent recovery of stress-tolerant plants.

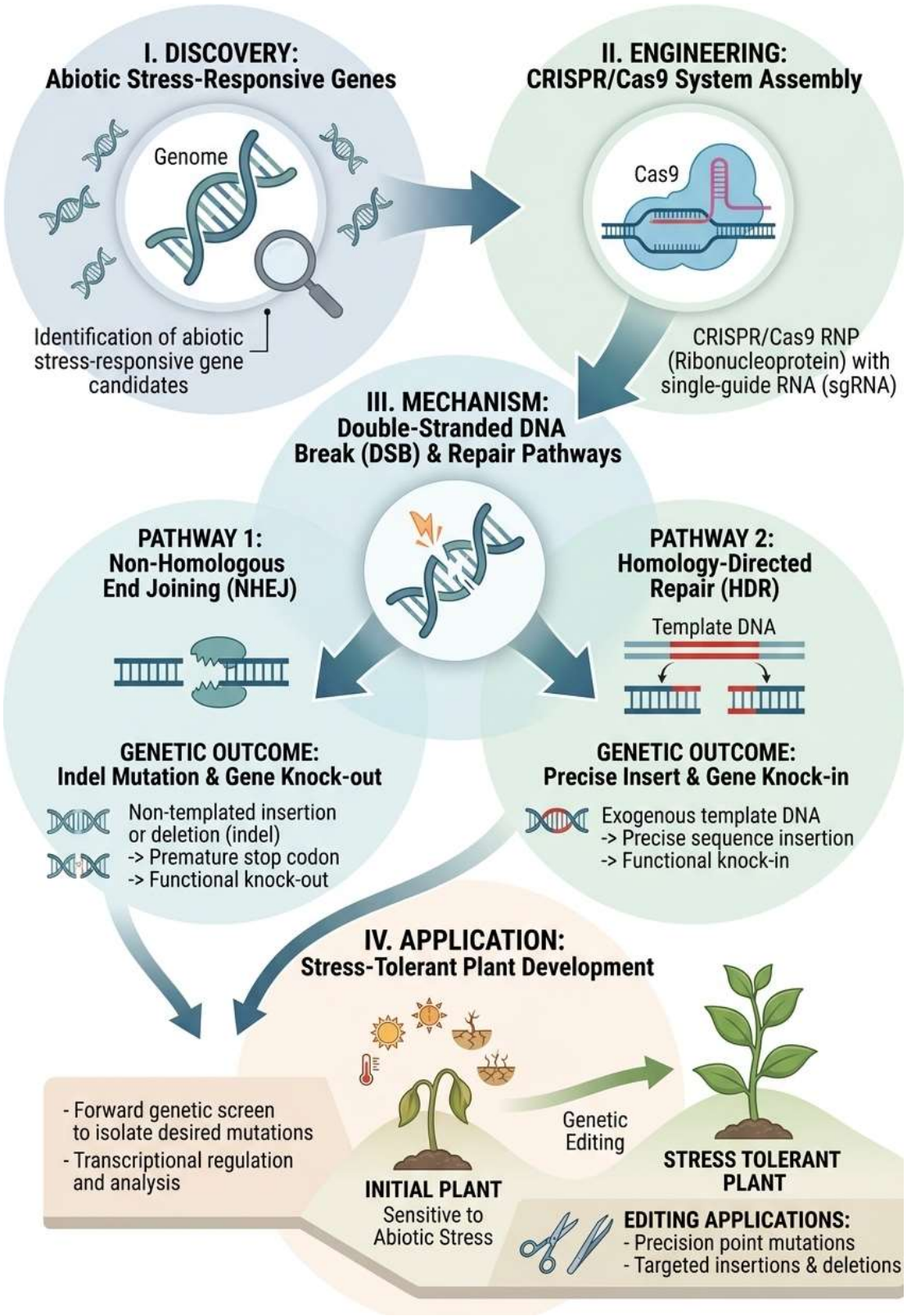


Figure 1: Schematic Workflow of CRISPR/Cas9-Mediated Engineering for Abiotic Stress Tolerance in Plants: From Target Gene Discovery to Trait Development.

2. CRISPR-Cas Toolkits and Delivery Mechanics in Rice Functional Genomics

The application of CRISPR-Cas platforms in rice functional genomics has rapidly expanded from basic single-gene targeted knockouts to sophisticated multiplexed editing, base editing, and prime editing systems. The canonical *Streptococcus pyogenes* Cas9 (SpCas9) nuclease remains the most widely deployed

genome editing tool (Khan et al., 2026). SpCas9 recognizes a 5'-NGG-3' protospacer adjacent motif (PAM) and generates blunt-ended double-strand breaks (DSBs) 3 base pairs upstream of the PAM site. These DSBs are predominantly repaired through the error-prone non-homologous end joining (NHEJ) pathway, introducing short insertion or deletion (InDel) mutations that disrupt open reading frames. To enhance targeting precision and mitigate off-target cleavage across the rice genome, high-fidelity Cas9 variants, such as eSpCas9 and SpCas9-HF1, have been engineered and optimized for monocot expression (Gica et al., 2026).

Complementing SpCas9, the Type V nuclease Cas12a (formerly Cpf1) broadens the scope of genomic targets by recognizing thymine-rich PAM sequences (5'-TTTV-3') and generating staggered DSBs with 4- or 5-nucleotide 5' overhangs. The cohesive sticky ends generated by Cas12a facilitate targeted gene insertion and homology-directed repair (HDR). Moreover, Cas12a processes its own short precursor CRISPR RNA (pre-crRNA) array, making it exceptionally efficient for multi-target editing from a single polycistronic transcript (Dash et al., 2025).

Promoter choice and expression vector design are critical for achieving high-efficiency editing in rice. Plant RNA Polymerase III promoters, such as the rice endogenous *OsU3* and *OsU6* promoters, drive sgRNA transcription with precise start sites at 'A' and 'G' nucleotides, respectively (Zhang et al., 2017). Multiplex expression architectures utilizing endogenous transfer RNA (tRNA) processing mechanisms or polycistronic Csy4 ribonuclease cleavage arrays allow the simultaneous editing of numerous distinct loci. This multiplexing capability is vital for dissecting polygenic abiotic stress traits and overcoming functional redundancy within multigene families, such as the 13-member *OsPYL* ABA receptor family (Li et al., 2026). Beyond targeted abiotic stress responses, modern genome editing toolkits enable broad phenotypic enhancements across diverse agronomic categories, as comprehensively summarized in **Figure 2**. This conceptual framework highlights multifaceted applications spanning biotic stress resistance, herbicide tolerance, hybrid breeding solutions, yield optimization, and physiological modulations for enhanced carbon accumulation and drought tolerance.

Delivery of CRISPR components into rice tissue is primarily executed via *Agrobacterium tumefaciens*-mediated transformation of embryogenic calli or biolistic particle bombardment. Additionally, direct transfection of pre-assembled Cas9 protein-sgRNA ribonucleoprotein complexes (RNPs) into protoplasts provides a transgene-free editing approach that circumvents foreign DNA integration. The high abundance of PAM sites across the rice genome (~1 in every 10 base pairs) facilitates rapid genome-wide screening using CRISPR knockout libraries (Sharma et al., 2026).

To systematically compare the diverse ribonucleoprotein platforms, nucleases, and cleavage architectures utilized in rice functional genomics, **Table 1** provides a detailed comparative overview. It outlines the nuclease types, PAM recognition sequences, cleavage patterns, and primary applications for standard SpCas9, high-fidelity variants, Cas12a, multiplex tRNA/U3 arrays, and transgene-free ribonucleoprotein delivery systems.

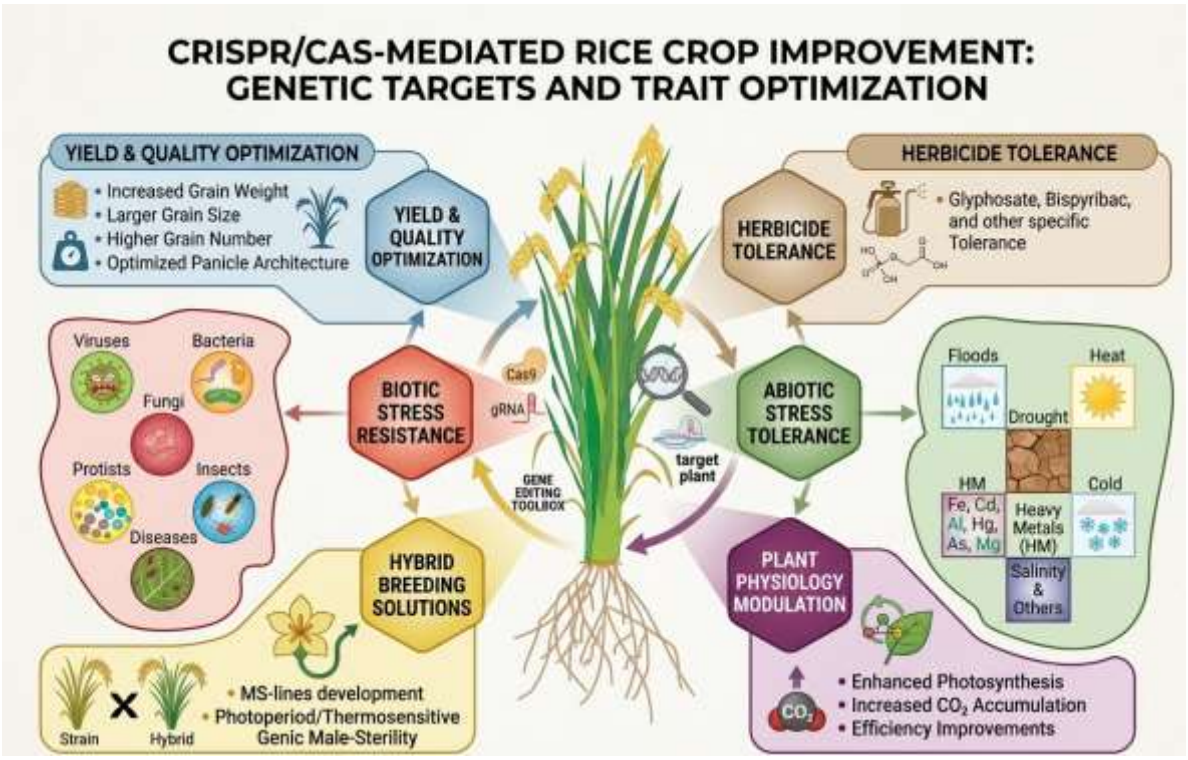


Figure 2: Comprehensive Overview of CRISPR/Cas Target Categories and Agronomic Trait Enhancements in Rice Crop Improvement.

Table 1. Overview of CRISPR-Cas Systems and Engineered Platforms Employed in Rice Stress Functional Genomics.

Tool / Platform	Nuclease Type	PAM Sequence	Cleavage Pattern	Primary Application in Stress Functional Genomics
SpCas9	Type II	5'-NGG-3'	Blunt DSB	Target gene knockouts, loss-of-function mutant libraries
High-Fidelity SpCas9	Type II (Variant)	5'-NGG-3'	Blunt DSB	Precision editing with reduced off-target mutations
Cas12a (Cpf1)	Type V	5'-TTTV-3'	Staggered DSB (5' overhang)	T-rich region targeting, multiplex editing, promoter modification
Multiplex tRNA/U3 Array	Type II / V System	Platform-dependent	Multi-locus DSB	Simultaneous knockout of multigene families and redundant loci
Cas9 RNPs	Ribonucleoprotein	5'-NGG-3'	Blunt DSB	Transgene-free editing via protoplast delivery

3. Functional Characterization of Genes Governing Root System Architecture (RSA)

Root system architecture defines the spatial arrangement, structural organization, and physiological distribution of roots within the soil profile. Rice root systems consist of a short-lived embryonic primary root, post-embryonic shoot-borne crown roots, and variable lateral root classes. Modulating RSA particularly transitioning from a shallow root growth angle to a steep, deep rooting angle enables plants to absorb water from deeper soil strata during surface soil drying, forming a foundational mechanism of drought avoidance (Kaur et al., 2026).

Polar Auxin Transport (PAT) provides the biochemical gradients that govern root gravitropism, primordia initiation, and cell elongation. Members of the PIN-FORMED (PIN) efflux carrier family and specific receptor-like kinases modulate local auxin distributions. targeted disruption of *OsPIN1b* using CRISPR-Cas9 highlighted the role of PAT in root structural development (Maqbool et al.,2022). Functional knockout of *OsPIN1b* (*ospin1b*) results in marked plant dwarfism, primary root growth inhibition, reduced

adventitious root counts, enlarged flag leaf angles, and light/culture-dependent root agravitropism. Disruption of PAT in *ospin1b* impairs root gravitropic setpoint angles, preventing deep root penetration (He et al., 2023).

In contrast, the leucine-rich repeat receptor-like kinase OsRPK1 acts as a negative regulator of PAT and root morphogenesis. Functional characterization of *OsRPK1* through CRISPR-Cas9 mutagenesis demonstrated that mutating this locus relieves PAT inhibition (Orman-Ligeza et al., 2014). In edited lines, primary root length, root depth, total root volume, and surface area increase significantly, accompanied by reductions in root diameter and root growth angle. This altered architecture enhances subsoil water extraction under dry conditions, translating to increased effective tiller counts, higher 1000-grain weight, and sustained grain yield under drought stress (Ren et al., 2022).

Root growth angle relative to the soil surface is further regulated by gravitropic response factors such as *DEEPER ROOTING 1* (*DRO1*) and its homolog *qSOR1* (*QUANTITATIVE TRAIL LOCUS FOR SOIL SURFACE ROOTING 1*). DRO1 expresses in root columella cells and promotes downward root bending in response to gravity. Optimizing *DRO1* expression converts shallow root structures into steep, deep-penetrating roots without altering shoot architecture, sustaining hydration during surface drought (Keerthi et al., 2026).

Beyond auxin transport, transcription factor networks govern crown root initiation and cell differentiation under environmental stress. The WUSCHEL-related homeobox transcription factor OsWOX11 serves as a master regulator integrating environmental signals, such as soil compaction and water deficit, with hormonal responses. Under physical stress or drought, elevated ethylene levels accumulate in root tissue, stabilizing OsEIL1, which transactivates *OsWOX11* expression (Wang et al., 2022). OsWOX11 interacts with ERF3 to repress type-A cytokinin response regulators like *OsRR2*, driving crown root primordia initiation. Furthermore, OsWOX11 maintains ROS homeostasis in root tips during water deficit by directly regulating peroxidase expression through the *OsWOX11-OsPRX130* module. CRISPR-generated loss-of-function *oswox11* mutants exhibit severe crown root initiation defects, higher water loss rates, and seedling survival rates under drought of only 5–15%, compared to 40% in wild-type plants. Overexpression of *OsWOX11* elevates survival rates up to 50–70% (Wu et al., 2022).

Targeted functional screens using CRISPR knockout libraries in *indica* rice identified additional root regulators, including *OsOPR5* (encoding 12-oxophytodienoate reductase involved in jasmonic acid biosynthesis) and *OsDRP1C* (a dynamin-related protein governing endocytosis). Knockout of *OsDRP1C* via CRISPR-Cas9 causes severe root stunting due to defective endocytic membrane trafficking and disrupted amino acid/glutathione metabolic fluxes (Chowdhury et al., 2026). A comprehensive summary of the specific CRISPR-Cas edited loci, protein classifications, mutation types, and their resulting root system architecture phenotypes is detailed in **Table 2**. This tabular synthesis underscores how precise genetic modifications in key regulators drastically alter root depth, growth angle, crown root initiation, and subsoil water capture capacity to confer drought avoidance. Similarly, OsDUF1223 positively regulates seedling root elongation through ABA and nitrogen signaling pathways. Additionally, OsNAC6 positively regulates root production by directly binding the promoter of nicotinamide synthase (*OsNAS1*), promoting nicotianamine accumulation and vascular development under stress (Ganie et al., 2024).

Table 2. Functional Roles of CRISPR-Cas Edited Loci Governing Root System Architecture (RSA) in Rice.

Target Gene	Protein Domain / Classification	Mutation / Edit Type	RSA Phenotype in Mutant Lines	Physiological & Yield Outcome Under Stress
OsPIN1b	Auxin efflux transporter	Frameshift InDel (1 & 4 bp del)	Shortened primary root; reduced adventitious roots; root agravitropism	Impaired root depth and altered gravitropic response
OsRPK1	LRR receptor-like kinase	Targeted exon mutagenesis	Increased root length, volume,	Superior subsoil water capture; higher tiller

			surface area; decreased root angle	number and seed weight
OsWOX11	WOX transcription factor	Target knockout (*oswox11*)	Severely suppressed crown root initiation; defective primordia emergence	Low survival under drought (5-15%); heightened water loss rate
OsDRP1C	Dynamin-related protein	Frameshift knockout	Stunted primary, adventitious, and lateral roots	Disrupted endocytosis and amino acid/glutathione metabolic flux
OsOPR5	12-Oxophytodienoate reductase	Library screen knockout	Altered lateral root density and JA-mediated root branching	Modulated root architectural development under stress
OsDUF1223	DUF1223 domain protein	Target knockout	Reduced seedling root elongation	Impaired ABA and nitrogen-mediated root extension
OsNAC6	NAC transcription factor	Target editing / knockout	Decreased crown root number and vascular bundle density	Lower drought avoidance due to reduced nicotianamine (*OsNAS1*)

4. Engineering Stomatal Patterning and Water Use Efficiency (WUE)

Stomatal gas exchange involves a physiological trade-off: stomata open to permit carbon dioxide (CO_2) uptake for photosynthesis (A_{net}), which inherently results in transpirational water loss (E). WUE, measured as biomass produced per unit of water transpired (A_{net}/E), can be optimized by adjusting stomatal density, size, and movement dynamics (Franks et al., 2015).

Epidermal Patterning Factor (EPF) signaling governs stomatal lineage development in rice. OsEPF1 acts as a negative regulator of stomatal development, whereas OsEPFL9 (STOMAGEN) acts as a positive competitive ligand that promotes stomatal differentiation in protodermal cells. CRISPR-Cas9 and CRISPR-Cas12a knockouts of *OsEPFL9* produced loss-of-function lines exhibiting up to an 80% reduction in leaf stomatal density (Chaerle et al., 2005). These low-density lines display reduced transpirational water loss, lower excised leaf water loss, and improved soil moisture retention during drought. However, severe reductions in stomatal density can limit maximum photosynthetic rate (A_{max}) under well-watered conditions due to reduced stomatal conductance (g_s), while decreasing evaporative canopy cooling (Ferguson et al., 2024).

To resolve this limitation, paralog editing of *OsEPFL9* or targeted editing of *OsEPF1* has been deployed to modulate stomatal density to intermediate levels. This approach balances stomatal density, maintaining photosynthetic assimilation during well-watered conditions while preserving higher leaf relative water content and improved intrinsic WUE (A_{net}/g_s) during water deficit (Dunn et al., 2019).

Stomatal density and movement kinetics are also regulated by specific zinc-finger transcription factors and ubiquitin ligases. The rice transcription factor, *OsDST* (*DROUGHT AND SALT TOLERANCE*) directly controls stomatal developmental gene networks. In *indica* rice varieties such as MTU1010, CRISPR-Cas9 targeted deletion within the *OsDST* locus (generating the *dstΔ184–305* mutation) downregulates core stomatal lineage genes, including *SPEECHLESS 1* (*OsSPCH1*), *MUTE*, and *INDUCER OF CBF EXPRESSION 1* (*OsICE1*) (Wang et al., 2004). The *dstΔ184–305* mutant lines exhibit lower stomatal density, faster ABA-induced stomatal closure, reduced transpiration water loss, and enhanced drought resilience. Furthermore, *OsDST* editing alters leaf anatomy, expanding bulliform cell size and reinforcing vascular bundle sclerenchyma, which prevents leaf rolling during dehydration (Bertolino et al., 2019).

Similarly, the RING-H2 E3 ubiquitin ligase OsDIRH2 functions as a negative regulator of drought responses. CRISPR-Cas9 knockout of *OsDIRH2* (derived from characterization of the TILLING line *ditl4*) yields plants with reduced stomatal density and heightened stomatal sensitivity to ABA. These edited lines conserve internal water, retain higher chlorophyll content, and accumulate greater dry biomass under drought conditions (Way et al., 2014). To elucidate the precise genetic interventions governing leaf gas exchange and transpirational control, **Table 3** compiles targeted genome editing approaches for stomatal development and water retention genes. It links specific modifications in secreted peptide ligands and zinc-finger transcription factors—such as *OsEPFL9* and *OsDST*—to their respective stomatal density reductions, anatomical changes, and overall water-use efficiency outcomes.

Table 3. Targeted Genome Editing of Stomatal Development and Water Retention Genes in Rice.

Gene Target	Protein Class	Editing Method	Stomatal & Anatomical Phenotype	WUE & Stress Physiology Outcome
OsEPFL9	Secreted peptide ligand	CRISPR-Cas9 / Cas12a KO	Up to 80% reduction in stomatal density	Decreased transpiration; conserved soil moisture under drought
OsEPF1	Secreted peptide ligand	CRISPR-Cas9 editing / OX	Tuned increase or decrease in stomatal density	Optimized carbon fixation and enhanced transpirational control
OsDST	C2H2 zinc-finger TF	CRISPR-Cas9 deletion (*dstΔ184–305*)	Reduced stomatal density via *SPCH1/MUTE/ICE1* repression	Reduced excised leaf water loss; reinforced vascular sclerenchyma
OsDIRH2	RING-H2 E3 ubiquitin ligase	CRISPR-Cas9 KO	Lower stomatal density; increased ABA sensitivity	Reduced transpiration rate; elevated biomass retention during drought

5. Dissecting Phytohormonal Cascades and Abscisic Acid Signaling Networks

Abscisic acid (ABA) is the core phytohormone governing physiological drought responses. The canonical ABA signaling cascade operates through a module consisting of PYR/PYL/RCAR receptors, PP2C phosphatase repressors, and SnRK2 protein kinases. Upon ABA binding, PYL receptors inhibit PP2Cs, releasing SnRK2 kinases to phosphorylate downstream transcription factors that activate stress-responsive genes (Kumar et al., 2019).

The rice genome contains 13 *PYL* gene family members (*OsPYL1* through *OsPYL13*), which show functional redundancy and distinct tissue-specific expression patterns. Dissecting their individual and combined roles was achieved using dual-vector multiplex CRISPR-Cas9 systems to systematically target Group I (*OsPYL1–6*, *12*) and Group II (*OsPYL7–11*, *13*) genes (Gomez-Cadenas et al., 2015).

Functional evaluation of multiplex edited lines revealed distinct physiological roles across the family:

- High-order Group I knockouts (e.g., *pyl1/2/3/4/5/6/12* septuple mutants) cause severe ABA insensitivity, leading to unclosed stomata, rapid water loss, and growth abnormalities.
- Lower-order targeted knockouts, such as the *pyl1/4/6* triple mutant, exhibit enhanced high-temperature tolerance, suppressed pre-harvest sprouting, and improved grain yields without sacrificing baseline drought survival (Singh & Roychoudhury, 2023).
- Targeted knockout of *OsPYL9* alone alters plant architecture, reduces water loss, modulates circadian rhythm and stress-responsive proteomes, and improves grain yield under field drought.
- Overexpression of monomeric receptors, such as *OsPYL/RCAR7*, enhances drought tolerance by accelerating ABA-mediated stomatal closure without inducing vegetative growth penalties (Chen et al., 2023).

Drought responses also involve intensive crosstalk between ABA and other phytohormonal pathways. The transcription factor OsNAC016 integrates brassinosteroid (BR) and ABA signaling pathways. Under optimal conditions, BR signaling promotes growth through OsBZR1; under drought stress, the BIN2

kinase stabilizes ABA-responsive transcription factors such as ABI5 while repressing BR signaling. OsNAC016 regulates this junction, balancing plant height, leaf inclination, and drought survival (Sarma et al., 2026).

Another NAC family member, OsNAC14, positively regulates drought tolerance by transactivating *OsRAD51A1*, a gene involved in double-strand DNA repair via homologous recombination. CRISPR-mediated knockout of *OsNAC14* (*osnac14*) downregulates *OsRAD51A1*, leading to accumulated genomic DNA damage, stunted root growth, and lower seed setting rates under stress. Conversely, *OsNAC14* overexpression lines maintain DNA repair efficiency, robust root growth, and seed filling during water deficit (Fàbregas et al., 2026).

Additional stress signaling regulators characterized via CRISPR editing include OsERA1 (ENHANCED RESPONSE TO ABA 1) and specific E3 ligases. Targeted mutagenesis of *OsERA1* confers hypersensitivity to ABA, promoting primary root growth under non-stressed conditions and inducing rapid stomatal closure upon dehydration, resulting in enhanced drought resistance (Romano, 2025).

Table 4 provides a structured breakdown of CRISPR-Cas dissection strategies applied to core phytohormone signaling loci and stress-responsive regulators in rice. By summarizing the signaling pathways, editing strategies, cellular biochemical effects, and agronomic stress outcomes of components like the *OsPYL* receptor family and *OsNAC* transcription factors, this table highlights the molecular basis of ABA-mediated drought resilience.

Table 4. CRISPR-Cas Dissection of Phytohormone Signaling Loci and Stress-Responsive Regulators.

Locus Target	Signaling Pathway / Mechanism	Editing Strategy	Cellular & Biochemical Effect	Agronomic Stress Outcome
OsPYL1/4/6	Group I ABA receptor module	Multiplex CRISPR KO	Moderate ABA insensitivity; altered stomatal closure	Increased high-temperature tolerance; lower pre-harvest sprouting
OsPYL9	ABA perception & circadian hub	Precise CRISPR KO	Proteomic shifts in stress & circadian proteins	Improved water retention and higher yield under field drought
OsNAC016	BR-ABA pathway crosstalk	Target gene editing	Modulates BIN2-OsBZR1-ABI5 regulatory axis	Balanced plant height, leaf angle, and drought survival
OsNAC14	DNA repair / HR pathway	CRISPR KO (*osnac14*)	Downregulates *OsRAD51A1*; elevated DNA damage	Reduced root depth and lower seed filling rate under stress
OsERA1	ABA response repressor	CRISPR mutagenesis	Enhanced sensitivity to endogenous ABA	Rapid stomatal closure; increased primary root elongation

6. Agronomic Trade-Offs, Multi-Trait Pyramiding, and Future Directions

Developing drought-resilient rice cultivars requires balancing physiological trade-offs associated with stress adaptations. Deep rooting angles (*DRO1*) and extensive lateral root systems (*OsRPK1*) enhance subterranean water access, but root growth incurs metabolic costs that can reduce shoot carbon allocation if not matched by adequate photosynthetic output (Li et al., 2026). Similarly, reducing stomatal density via *OsEPFL9* or *OsDST* edits conserves soil water and maintains leaf relative water content, but lower stomatal conductance (g_s) can reduce intercellular CO_2 concentration (C_i), suppressing peak photosynthesis (A_{net}) and reducing evaporative canopy cooling during combined heat and drought events (Mbebi et al., 2025).

To resolve these trade-offs, multi-trait gene pyramiding using multiplex CRISPR platforms offers a path forward. Simultaneously targeting negative regulators across independent pathways such as combining

stomatal density tuning (*OsEPF1 / OsDST*), root architecture optimization (*OsRPK1*), and yield-limiting genes (*GS3*, *GW2*) enables the uncoupling of stress tolerance mechanisms from yield penalties (Song et al., 2026).

Advancements in genome editing technologies further expand these capabilities:

1. **Promoter and Cis-Regulatory Element Editing**: Targeted editing of non-coding promoter regions using Cas9 or Cas12a generates quantitative expression gradients, allowing fine-tuned gene expression rather than complete gene knockouts (Zhou et al., 2026).
2. **Base Editing and Prime Editing**: Cytidine Base Editors (CBEs), Adenine Base Editors (ABEs), and Prime Editors (PE) enable precise single-nucleotide transitions without creating double-strand breaks. This allows modification of regulatory amino acid residues (e.g., phosphorylation sites on SnRK2s or ABA receptors) to alter enzyme activity or binding affinity (HABDE et al., 2026).
3. **Transgene-Free Editing Delivery**: Delivering Cas9-sgRNA ribonucleoprotein complexes (RNPs) via protoplasts, or segregating out transgene insertions in subsequent generations, yields edited rice lines free of foreign DNA. Transgene-free edited crops face reduced regulatory requirements, facilitating commercial deployment (Wang et al., 2026).

Field trials under variable multi-stress conditions (such as combined drought, salinity, and high temperature) remain essential. Integrating CRISPR-based functional genomics with marker-assisted selection (MAS) and genomic selection (GS) will accelerate the development of high-yielding, drought-avoiding, and water-use-efficient rice cultivars suited for climate-resilient agriculture (Xu et al., 2026).

Conclusion

CRISPR-Cas technology has fundamentally transformed the functional characterization of drought-adaptive genes in rice, enabling precise, targeted dissection of the complex genetic networks governing root system architecture, stomatal-mediated water use efficiency, and phytohormonal stress signaling. By overcoming the limitations of conventional forward genetics including linkage drag, functional redundancy, and labor-intensive gene identification CRISPR platforms have illuminated the individual and combinatorial roles of key loci such as *OsPIN1b*, *OsRPK1*, *DRO1*, *OsWOX11*, *OsEPFL9*, *OsDST*, and the *OsPYL* family. These studies reveal that drought resilience is a polygenic trait requiring coordinated regulation of water acquisition, conservation, and cellular protection. However, physiological trade-offs persist: enhanced deep rooting and reduced stomatal density can compromise shoot carbon allocation and photosynthetic capacity under combined heat and drought. Multiplex editing and multi-trait pyramiding simultaneously targeting negative regulators of RSA, stomatal development, and yield-limiting genes offer a promising route to uncouple stress tolerance from yield penalties. Emerging tools, including base and prime editing, promoter/cis-regulatory element editing, and transgene-free ribonucleoprotein delivery, will further refine trait optimization and accelerate regulatory approval. Future efforts must integrate CRISPR functional genomics with marker-assisted selection, genomic selection, and rigorous multi-stress field trials to deliver high-yielding, water-use-efficient, climate-resilient rice cultivars capable of sustaining global food security.

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