

Engineering drought and salinity tolerance traits in crops through CRISPR-mediated genome editing: Targets, tools, challenges, and perspectives

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ABSTRACT

Prolonged periods of drought triggered by climate change hamper plant growth and cause substantial agricultural yield losses every year. In addition to drought, salinity is one of the major abiotic stresses that severely affect crop health and agricultural production. Plant responses to drought and salinity involve multiple processes that operate in a spatiotemporal manner, such as stress sensing, perception, epigenetic modifications, transcription, post-transcriptional processing, translation, and post-translational changes. Consequently, drought and salinity stress tolerance are polygenic traits influenced by genome-environment interactions. One of the ideal solutions to these challenges is the development of high-yielding crop varieties with enhanced stress tolerance, together with improved agricultural practices. Recently, genome-editing technologies, especially clustered regularly interspaced short palindromic repeats (CRISPR) tools, have been effectively applied to elucidate how plants deal with drought and saline environments. In this work, we aim to portray that the combined use of CRISPR-based genome engineering tools and modern genomic-assisted breeding approaches are gaining momentum in identifying genetic determinants of complex traits for crop improvement. This review provides a synopsis of plant responses to drought and salinity stresses at the morphological, physiological, and molecular levels. We also highlight recent advances in CRISPR-based tools and their use in understanding the multi-level nature of plant adaptations to drought and salinity stress. Integrating CRISPR tools with modern breeding approaches is ideal for identifying genetic factors that regulate plant stress-response pathways and for the introgression of beneficial traits to develop stress-resilient crops.

Key words: CRISPR/Cas, drought tolerance, genome editing, polygenic traits, salt tolerance, trait introgression

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INTRODUCTION

Agricultural scientists are expected to face several challenges in the coming years. Some examples include the design of climate-resilient crop varieties, understanding the effects of plant-environment interactions on genetic adaptations, improving crop yields, linking plant genotype to phenotype, and developing efficient high-throughput plant phenotyping methods (Varshney et al., 2021a). Increasing human populations and climate change are the primary obstacles to meeting global food and

energy demands proposed as sustainable development goals by the United Nations. Climate change triggers dramatic shifts in water availability and distribution via aberrations in seasonal precipitation, evaporation, and weather patterns; such factors cause imbalances in the salinity and nutrient availability of

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cultivated soils at global and regional levels (Cai et al., 2014; Konapala et al., 2020). The accretion of these factors gradually transforms once-fertile lands into problematic to barren lands. Ultimately, such events impede crop productivity and commercial agriculture around the world. In addition to climate change, several inanimate factors contribute to abiotic stress in plants, such as lack of water availability over a period of time, sudden or prolonged exposure to high or low temperatures, and higher salt and heavy metal content in the soil (Zafar et al., 2020). Changing rainy seasons will also alter the magnitude and recurrence of drought-like situations, significantly affecting agricultural productivity (Cai et al., 2014). Farmers lose more crop yields annually from drought alone than from all pathogens (Gupta et al., 2020).

Salinity stress ranks second to drought in terms of global constraints that limit crop productivity and land use for agriculture (Gong et al., 2020). About one-fifth of the irrigated land around the globe is estimated to be affected by higher salt accumulation (Morton et al., 2019). In a broad sense, water scarcity and soil salinization are inter-linked processes (Zhu, 2002). Salinization of naturally occurring non-saline soils increases because of irrigation practices and climate change-triggered rising sea levels or higher evaporation rates (Van Zelm et al., 2020). Moreover, the excessive use of chemicals and fertilizers has led to deterioration in soil health by increasing salt accumulation. Such problematic soils are increasing, with a minimum of 10% annual growth; to exacerbate this situation, about half of presently cultivated soils may become salinized by 2050 (Jamil et al., 2011). Overall, drought and salinity are major abiotic factors that severely affect agricultural yields. Therefore, to meet burgeoning food and energy demands, engineering of drought- and salt-tolerant crop varieties is essential under the impeding global warming and climate change conditions.

Plant scientists strive to develop crop varieties with stress-adaptive traits that thrive under changing climates (Henry, 2020). Such climate-smart crops will ensure a steady supply of food and energy products. The stacking of beneficial alleles in elite varieties was achieved through breeding in the past. Hybridization, mutation breeding, and improved agronomic practices increased global food production during the 1960s, leading to the “green revolution.” These traditional approaches are still helpful for developing varieties resilient to changing environmental conditions (Shelake et al., 2019a). However, conventional breeding methods are cumbersome, time consuming, and often fail to produce the desired outcomes, specifically with regard to abiotic stress tolerance, owing to complex genetic inheritance, pleiotropic effects, and unpredictable gene-environment interactions (Varshney et al., 2021b). In the 1990s, transgenic technology and RNA interference were used to produce genetically modified (GM) varieties with enhanced traits such as insect pest resistance, herbicide tolerance, abiotic stress or disease resistance, and biofortification (Singh et al., 2009; Kumar et al., 2020). However, GM varieties are subject to regulatory hurdles and have limited applications (Ahmad et al., 2021).

Recent advances have enabled the generation of targeted mutations at desired loci in plant genomes (Shelake et al., 2019a). For instance, modern breeding techniques include genome editing

(GE) tools that enable fast-track introgression of novel beneficial traits into crop varieties; these approaches are commonly referred to as new plant breeding technologies (NPBTs). Modern breeding techniques can be complemented by use of the latest NPBT approaches. Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (CRISPR/Cas) is the newest addition to the GE arsenal (Jinek et al., 2012). The engineered CRISPR/Cas system can enable editing of a desired locus in a complex genome. CRISPR-based tools have been used effectively for genome engineering in model plants and crop species in a range of fundamental to applied research (Pramanik et al., 2021). Technical breakthroughs in CRISPR-based approaches have made it possible to create transgene-free GE plants. Newer regulations are being formulated to handle the commercial release of GE products, including crop varieties deemed non-GM crops, by consideration on a case-by-case basis (Menz et al., 2020). Recently, genomic-assisted breeding (GAB) and CRISPR-based GE studies have shown promising outcomes in the exploration of plant stress-response pathways.

Moreover, CRISPR tools contribute to discovering the inherent molecular aspects of drought/salinity stress tolerance in plants (Joshi et al., 2020a; Zafar et al., 2020; Ahmad et al., 2021). This review provides an overview of plant responses to drought and salinity stresses at the molecular, physiological, and morphological levels. We discuss CRISPR-based techniques, CRISPR applications for basic and applied research on drought and salinity, and crucial aspects of developing stress-adaptive transgene-free GE varieties by combining genomics-assisted breeding approaches.

PLANT RESPONSES TO DROUGHT STRESS

Drought and salinity stress tolerance are polygenic traits. Plants have evolved several ways to tackle stressful events, which involve the activation of overlapping pathways at morphological, physiological, and molecular levels in response to different abiotic stresses. In addition, various abiotic stresses share some common characteristics of stress perception and effects on plant growth or development. For example, abiotic stresses like drought or salinity usually trigger osmotic stress in plant cells. Several components are often associated with more than one type of abiotic-stress-related pathway; these include sensors, receptors, phytohormones, transcription factors (TFs), kinases, phosphatases, and microRNAs (miRNAs) (Gong et al., 2020; Hussain et al., 2021). Drought stress is instigated by low atmospheric and soil humidity under higher ambient temperatures following water deficit for normal plant growth (Lipiec et al., 2013). Plants adapt their water balance dynamically under drought conditions to reduce water loss or improve water uptake (Mickelbart et al., 2015; Lamaoui et al., 2018; Joshi et al., 2020a; Varshney et al., 2021b).

Drought stress perception and tolerance are complex quantitative phenomena governed by multiple genes and biochemical pathways at various stages of plant life progression (Bhat et al., 2020). The multi-level processes include sensing, signaling, and molecular changes to various layers of the central dogma, such

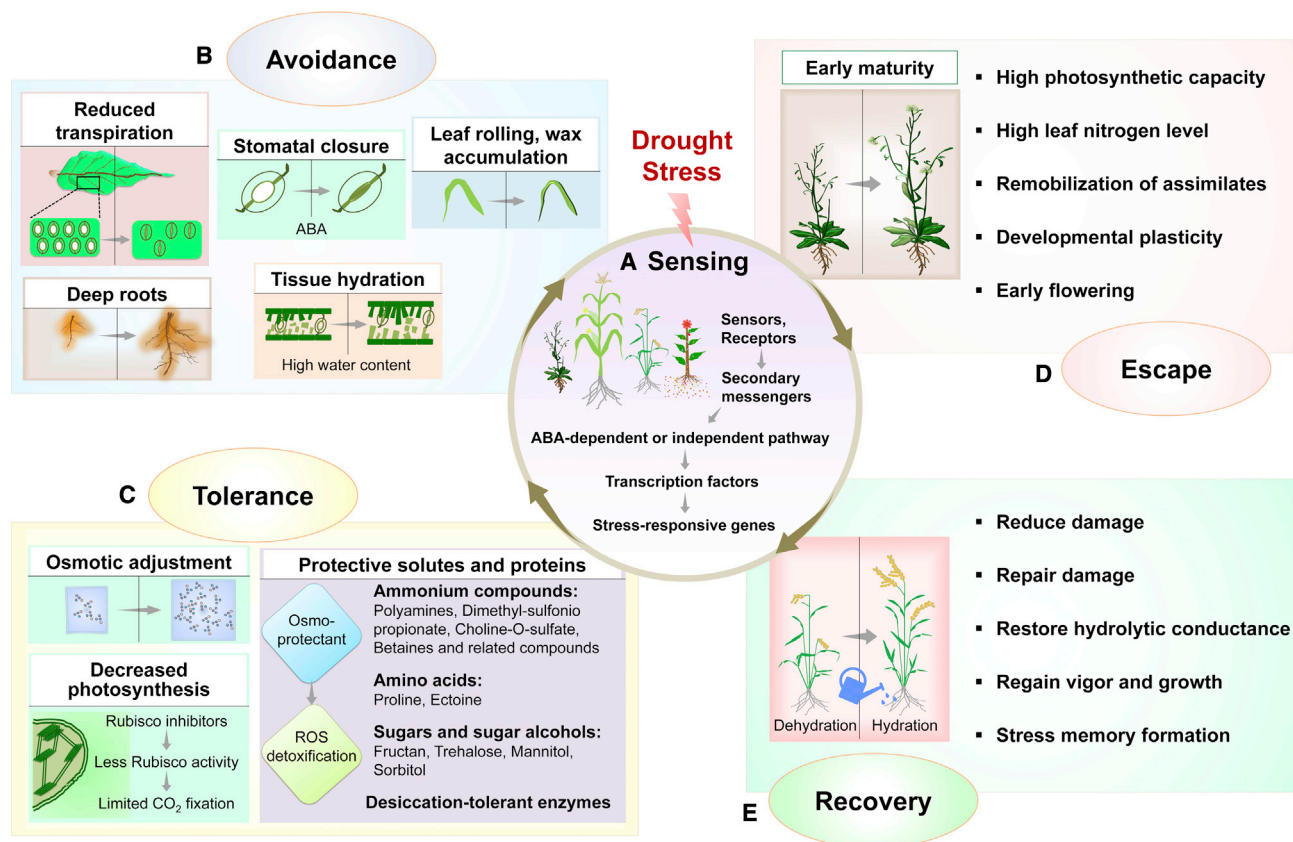


Figure 1. Plant responses to drought stress.

(A–E) Four critical processes involved in plant drought-stress tolerance after sensing the stress (A) are depicted, along with major changes used by plants in each of the processes, which include avoidance (B), tolerance (C), escape (D), and recovery (E). Specific plant species may respond to drought stress differently, and plants use more than one type of process for growth and survival.

as epigenetic modification, transcription, translation, and protein post-translational modifications (Zhang et al., 2021). Once a plant senses drought stress, plant responses can be grouped into four main categories: avoidance, tolerance, escape, and recovery (Figure 1).

An encounter with any stress activates cellular response pathways by altering the physical or chemical state of biomolecules in the cell. Although assigning the role of stress sensor to a particular molecule is complex, some are characterized using indirect approaches. Different primary drought-sensing modes have been suggested in the past (Zhang et al., 2021). For example, a mutant library of putative sensors is expected to alter the production of secondary messengers like Ca^{2+} , reactive oxygen species (ROS), nitric oxide, and phospholipids, enabling screening of the resultant phenotypes. Such an approach has been applied in *Arabidopsis* to find the first putative osmotic stress sensor, *OSCA1*, a plasma membrane (PM) protein that forms Ca^{2+} -permeable channels under hyperosmotic conditions (Yuan et al., 2014). Subsequently, several members of the *OSCA* family have been identified in different plants, including 15 in *Arabidopsis* (Yuan et al., 2014), 11 in rice (Li et al., 2015), 12 in maize (Ding et al., 2019), 13 in mung bean (Yin et al., 2021), and 42 in wheat (Tong et al., 2022). The *OSCA* family members may be functionally redundant, and further characterization would be the starting point to exploring the role of *OSCA* homologs in

different stress-response pathways. Drought avoidance is the water-saving strategy that plants adopt under water-deficit conditions (Ilyas et al., 2021). Drought avoidance is achieved through morpho-physiological changes that involve stomatal regulation, limited vegetative growth via a reduced leaf number or leaf area, wax accumulation, an enhanced root system to take up available water, and avoidance of dehydration by lower water use.

Drought tolerance is the ability of a plant to thrive under severe water-deficit conditions through the modulation of physiological processes at different developmental stages (Fang and Xiong, 2015). One or more combinatorial drought tolerance strategies used by plants include low stomatal density, activation of stress-responsive genes, and alteration of signal transduction and metabolic pathways. Osmoprotectants such as amino acids, ammonium compounds, and sugar molecules help with ROS detoxification and maintenance of osmotic pressure, leading to a drought tolerance phenotype (Singh et al., 2015a). Water-deficit conditions also decrease photosynthetic rate and limit assimilate partitioning, presumably owing to enhanced acid invertase activity (Kim et al., 2000). Phytohormones like abscisic acid (ABA) and gibberellin (GA) mediate the signaling cascades that stimulate stress-related gene expression triggered by TFs, rapid stomatal closure, and changes in plant development under drought, salt, and other abiotic stress conditions

(Cutler et al., 2010; Colebrook et al., 2014; Roca Paixão et al., 2019; Illouz-Eliaz et al., 2020; Wu et al., 2020).

The mechanism of stomatal opening is regulated via ABA and ABRE, involving a network of TFs. TFs from diverse families, for example, dehydration responsive element binding (DREB), bZIP, WHY, AP2/ERF, HD-Zip, bHLH, AREB/ABF, MYB, NAC, and WDR TFs, play multiple roles in drought stress tolerance and represent a crucial avenue for the development of drought-tolerant plant varieties (Joshi et al., 2016; Goel et al., 2019). Drought escape refers to plant adaptation to water deficit by shortening the life cycle or growth period. Flowering time is an ideal adaptive trait for drought-escape conditions. A transcriptome and phenotypic selection approach identified *OsMADS18* (a MADS-box TF) as a drought-escape gene, highlighting the importance of early flowering in drought escape (Groen et al., 2020). Farmers prefer plant varieties with short lifespans that can escape climatic drought conditions.

Some plant genotypes survive the drought period and resume their vigor and growth; this ability is called drought recovery. Plants develop drought stress memory during repeated stresses, allowing them to respond quickly and provide enhanced protection (Jacques et al., 2021). The development of stress memory involves epigenetic changes and physiological adaptations that alter photosynthesis, cellular protective functions, osmotic adjustment, energy mechanisms, maintenance of water status, and plant interactions with soil microbes.

PLANT RESPONSES TO SALINITY STRESS

Excessive salt accumulation in plant cells causes salinity stress, hindering growth and development (Singh et al., 2021). During salt stress, an imbalance between cytosolic free sodium (Na^+) and potassium (K^+) ions is the main factor linked with ion toxicity. Higher salt ion concentrations in the soil limit water and nutrient uptake owing to hyperosmotic stress (Lamers et al., 2020; Van Zelm et al., 2020). Consequently, plant productivity is hampered by reduced photosynthesis, leading to agricultural losses. To survive in highly saline soils, plants have evolved mechanisms that reduce ion uptake in roots, compartmentalize excess ions inside the cell, and adjust osmotic pressure (Mickelbart et al., 2015). Critical aspects of plant responses to salinity stress are emphasized in Figure 2.

Similar to drought, plant responses to surplus salt involve adaptations at multiple levels, such as morphological (lateral shoot growth inhibition, early flowering, and root modifications), physiological (ion sequestration, osmotic adjustment, stomatal conductance, Na^+/K^+ discrimination), and biochemical (accumulation of polyamines, sugars, and proline, changes in hormone levels, induction of antioxidant machinery and metabolic pathways for detoxification of cytotoxic compounds) adaptations. Key aspects of plant responses to salt stress involve sensing excessive salt, early Ca^{2+} and ROS signaling cascades that activate various stress-responsive genes to avoid cell damage, and loading the resulting signaling molecules for long-distance transport via apoplastic and symplastic pathways across different tissues (Figure 2). The perception of salinity stress, i.e., sensing

higher salt (Na^+/K^+) concentrations, is arguably the least understood phenomenon despite recent advances in plant salt-stress biology (Van Zelm et al., 2020). Recently, the *MOCA1* gene encoding a glucuronosyltransferase for glycosyl inositol phosphorylceramide (GIPC) sphingolipids, PM-localized proteins, was revealed to be a vital plant-specific salt sensor (Jiang et al., 2019).

Initially, Na^+ uptake is facilitated by transporters and channels such as nonselective cation channels (NSCCs), cyclic nucleotide-gated channels (CNGCs), glutamate receptors (GLRs), and high-affinity K^+ transporters (HKTs) (Tester and Davenport, 2003; Hanin et al., 2016; Qiu et al., 2020a, 2020b). Similarly, K^+ influx from the apoplast into the cytoplasm is carried out by channels like the *Arabidopsis* K^+ transporter (AKT1) and high-affinity K^+ uptake transporter (HAK). Plant cells recruit Ca^{2+} into the cytoplasm by CNGC and GIPC-interacting channels to deal with hyperaccumulated Na^+/K^+ ions (Hanin et al., 2016; Zhang et al., 2021). Along with Ca^{2+} , ROS and 3',5'-cyclic guanosine monophosphate (cGMP) constitute the three major salt-signaling molecules that activate salt-response pathways (Van Zelm et al., 2020).

After perceiving salt stress, early signaling responses comprise activation of Ca^{2+} signaling, ion transport, ROS-mediated signaling cascades, phospholipid modifications, and higher protein kinase activities (Chourasia et al., 2021). The process of Ca^{2+} signaling involves changes in Ca^{2+} spikes at three levels: instant at the cellular level (as early as 10 s after salt application) and fast- and late-response Ca^{2+} waves (Choi et al., 2014; Van Zelm et al., 2020). As depicted in Figure 2, Ca^{2+} binding to calcineurin B-like proteins (CBLs) instigates protein phosphorylation via a signaling cascade mediated by Ca^{2+} -dependent protein kinases (CDPKs) and CBL-interacting protein kinases (CIPKs). Different CBL-CIPKs have been found to coordinate various cellular pathways once activated by Ca^{2+} and ROS signaling (Manishankar et al., 2018). The salt overly sensitive (SOS) pathway is the best-characterized CBL-CIPK pathway involved in plant salt tolerance (Qiu et al., 2020a, 2020b; Van Zelm et al., 2020; Chen et al., 2021b). In brief, after perception of salt stress, a spike of Ca^{2+} is generated in the cytoplasm of root cells and activates the SOS signaling pathway. SOS3 (CBL4) becomes activated after binding with Ca^{2+} and interacts with SOS2 (CIPK24). The SOS3/SOS2 complex then stimulates SOS1 (NHX7) through phosphorylation in the presence of Ca^{2+} . SOS1 is a membrane-localized Na^+/H^+ antiporter in shoot and root cells (Halfter et al., 2000). Stele-localized SOS1 exports Na^+ out of the cell in exchange for H^+ at the root-soil interface, providing the first line of salt resistance (Shi et al., 2002). Elevated Na^+ is transported to the vacuole by NHX antiporters for storage. Later, the excess Na^+ and K^+ ions are pumped out of the cytoplasm back into the apoplast by SOS1 and K^+ outward rectifying channel (KOR) transporters, respectively.

Long-distance signaling with different molecular players and pathways leads to salinity tolerance by producing osmo-protectant proteins, metabolites, osmolytes, and transporters, regulating stomatal conductance, modifying the cell wall, and reorganizing root architecture. Plants use two pathways to reduce ion toxicity based on either restricting entry into plant cells via roots or sequestering inter- and intracellular ions into

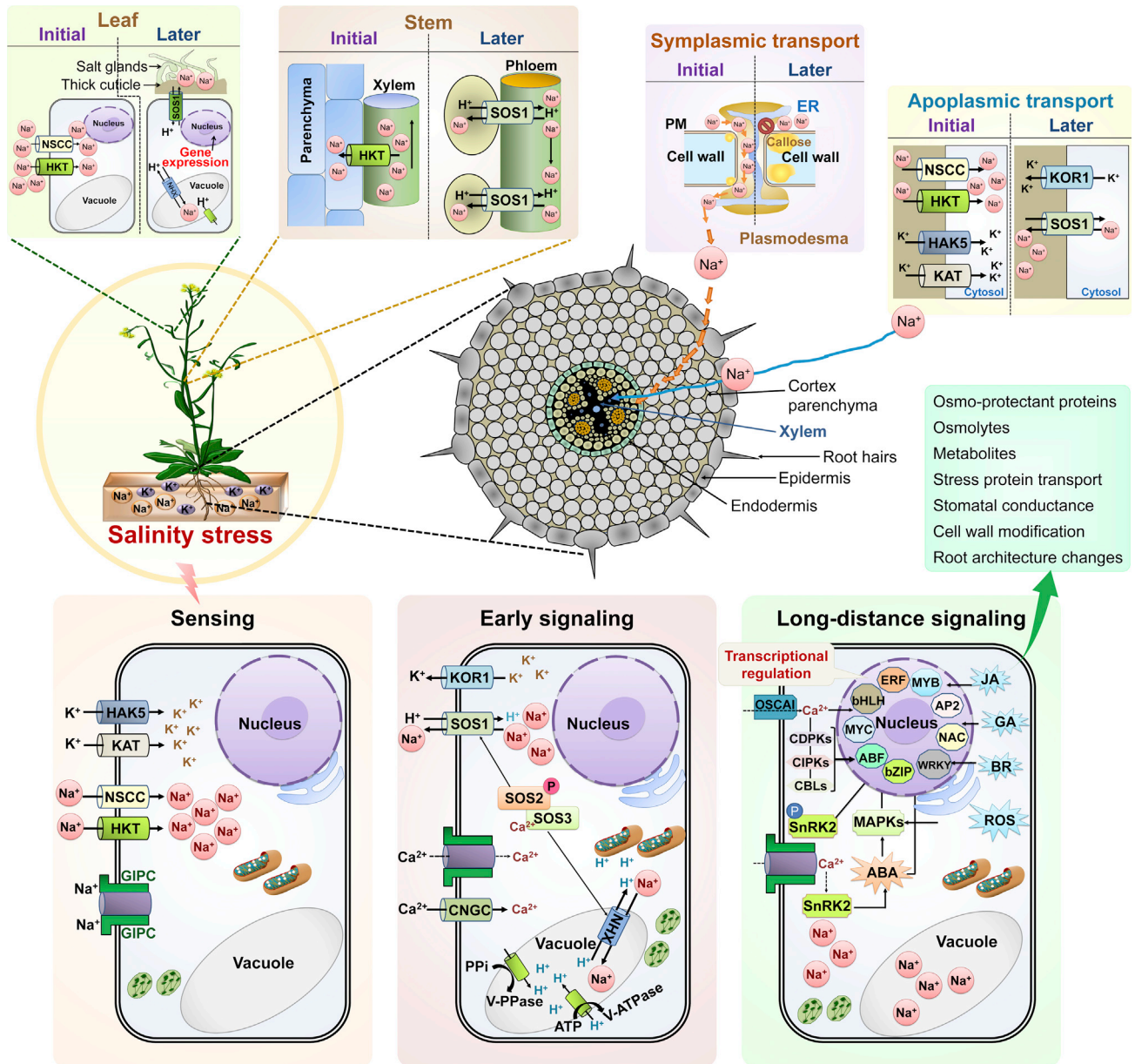


Figure 2. Overview of salinity stress sensing, signaling, and response in plants.

Salinity stress is mainly caused by toxic sodium (Na^+), potassium (K^+), and chloride (Cl^-) ions from sodium chloride (NaCl) and potassium chloride (KCl). In the current model, plant sensing, signaling, and response in the context of Na^+ and K^+ cytotoxicity are illustrated. Descriptions or full forms of the TF, gene, and family abbreviations are provided in the main text and supplemental information.

compartmentalized bodies such as vacuoles (Lou et al., 2018). Na^+ ions enter the root system by either the symplastic pathway or apoplastic transport. Na^+/K^+ homeostasis is a crucial factor for plant survival that is facilitated by transporters, antiporters, and channels under salt-stress conditions. Recently, several components of salt-homeostasis pathways across plant cells, tissues, and organs have been characterized. These components include protein kinases (CIPK, CDPK), TFs (NAC, WRKY, SERF, bHLH, bZIP, MYB, YABBY, MADS-box, HD-Zip), symporters, SOS antiporters, NHX exchangers, HKT transporters and carrier proteins, and cellular detoxification pathways (glyoxalase pathway) (Mustafiz et al., 2011; Gupta et al., 2018; Van Zelm et al., 2020; Hussain et al., 2021; Sun et al., 2021). Components of

the response to salinity and other stresses have been reviewed in recent literature, including summaries of protein kinase-mediated signal transduction (Chen et al., 2021b), interactions of TFs with ABA-dependent and -independent pathways during salt stress (Hussain et al., 2021), and the role of ion transporters in plant growth and stress responses (Tan et al., 2022).

Ca^{2+} is a leading player in long-distance signaling. Ca^{2+} enters the cytoplasm via OSCA1-like transporters or GIPC-interacting channels, thereby directly or indirectly regulating the activity of TFs involved in salinity tolerance via a cascade of CBLs, CIPKS, and CDPKs. ABA is one of the critical phytohormones involved in salt-stress defense; it integrates and coordinates

signaling during salinity stress and activates whole-plant-level changes involving plant metabolic pathways, ion transport, and developmental processes (Yu et al., 2020). ABA signaling directly influences the activities of TFs or acts via the MAPK signaling cascade that regulates the expression of genes involved in salt-stress mechanisms. Salinity stress is also accompanied by elevated production of ROS (singlet oxygen, superoxide, hydroxyl radicals, and hydrogen peroxide), which modulates the transcription of salt-responsive genes. Elevated levels of plant hormones like jasmonic acid (JA), gibberellic acid (GA), and brassinosteroids (BR) have been found to control TF activities directly or indirectly during long-distance signaling.

The SNF1-related protein kinases (SnRKs) are plant-specific enzymes that play a crucial role in modulating ABA-dependent and ABA-independent signaling pathways. Ca^{2+} -dependent activation of SnRK2 by osmotic and salt stress regulates ABA signaling (Fàbregas et al., 2020; Chen et al., 2021b). ABA-nonactivated kinases (SnRK2.4 and SnRK2.10) are activated in response to salt stress and affect the plant morphology of *Arabidopsis* (Mazur et al., 2021). Recent studies have shown that cytokinins are also associated with tolerance to abiotic stresses, including salinity (Wang et al., 2019). When plants are subjected to abiotic stresses, genes related to cytokinin signaling are spatiotemporally expressed, possibly connecting local signals to systemic plant responses (Zwack and Rashotte, 2015; Joshi et al., 2018). Overall, the role of phytohormones in abiotic stresses is not yet fully understood, owing to the complex network of associated pathways. In the later phase of the salt-response mechanism, salt-induced signaling pathways cause adaptive changes. Produced solutes mediate osmotic balance and modification of growth and developmental processes at tissue, organ, and whole-plant levels (Singh et al., 2021).

Salt transport occurs through both symplastic and apoplastic routes in root and shoot systems. Symplastic transport occurs through the plasmodesmata (Picard, 2003), and apoplastic transport is mediated by ion-specific transporter proteins (Hall et al., 2006; Munns et al., 2012). Plasmodesmata are symplastic nanochannels that connect adjacent cells and allow the passive or active movement of several molecules, including Na^+ ions (Iswanto et al., 2021). After activation of stress signaling, callose accumulation closes these nanochannels and regulates ion movement. On the other hand, Na^+ and K^+ transporters load ions into the xylem from the root cells for further apoplastic translocation towards the stem and leaves. The process of Na^+ loading/unloading in the xylem is a critical aspect of the salt-stress response mechanism. With the progression of salinity stress and subsequent signaling, ions are pumped out of cells through SOS1 and KOR1 in different tissues. Na^+ ions are unloaded into the xylem parenchyma cells by HKTs and other transporters. Antiporters like SOS1 flush them out into the phloem while unloading. In leaves, NSCC and HKT family members participate in Na^+ movement into the cell. Plants have evolved unique epidermal structures like salt glands and a thick cuticle layer to maintain ion homeostasis (Litalien and Zeeb, 2020). Excess Na^+ ions are secreted into the salt glands for storage and exclusion in some species, like halophytes. Overall, targeting plant genes that transduce early signals to activate downstream genes involved in stress signaling would enable the engineering of stress-resilient crop varieties.

CRISPR-BASED GENOME ENGINEERING TOOLS

Basics of the CRISPR/Cas system

Prokaryotes, including bacteria and archaea, contain a CRISPR array acquired as part of their immune system against viruses. The components of native CRISPR/Cas machinery have been repurposed for targeting desired genomic locations and precision editing (Cong et al., 2013; Mali et al., 2013). The engineered CRISPR/Cas9 system consists of two main components: endonuclease Cas9 enzyme and RNA (Figure 3A).

The optimized RNA component, also called single guide RNA (sgRNA), is a combination of CRISPR RNA (crRNA) and a *trans*-activating RNA (tracrRNA). The crRNA includes an RNA fragment of about 20 bp, a complementary sequence of the targeted site, and is usually cloned at the 5' end of the sgRNA. Recognition of the sgRNA-binding region and protospacer adjacent motif (PAM; typically 3–5 bp) in the genome is followed by multimolecular structural reorganization between the protein-RNA-DNA complex; i.e., Cas-sgRNA-target DNA (Nishimasu et al., 2014). Two endonuclease domains of the Cas enzyme cleave the DNA and generate double-stranded breaks (DSBs) in the targeted region, leading to programmable editing. The ultimate advantages of CRISPR-based GE technology are delivery of CRISPR components at the desired locus in the complex genomic architecture and highly efficient precision editing. Multiplex editing (simultaneously targeting multiple loci) is another feature of CRISPR and other GE tools that enables the engineering of polygenic or multiple traits (Clasen et al., 2016; McCarty et al., 2020).

Cas9 variants and orthologs with novel properties

The most commonly used Cas orthologs are Cas9 and Cpf1 (Cas12a). Cas9 from *Streptococcus pyogenes* (SpCas9) recognizes NGG as a PAM and produces blunt-end DSBs; it is the most commonly used Cas for animal and plant genome engineering (Figure 3A). Cas12a requires only a crRNA that recognizes the T-rich PAM site, and cleavage generates staggered-end DSBs (Zetsche et al., 2015). Point mutations in SpCas9 and Cas12a have created several variants with higher precision or relaxed PAM requirements (Figure 3B). Apart from SpCas9 and Cas12a, several Cas orthologs from the microbial community have been optimized for GE, recognizing distinct PAMs, displaying different editing specificities, and targeting RNA or DNA (Figure 3C). An RNA-guided RNA-targeting enzyme such as Cas13 enables programmable RNA editing (Abudayyeh et al., 2016). Overall, the growing CRISPR toolbox, including reagents with distinct features, has broadened the scope of crop genome engineering at different layers of the central dogma responsible for various plant traits or phenotypes.

CRISPR-based tools for gene, chromosome, and genome engineering

Several CRISPR-based tools have been developed in the last 10 years, enabling desired editing in the targeted genomic loci and beyond (Pramanik et al., 2021). Some examples include DNA base editors (BEs), epigenetic modifiers, prime editors (PEs), transcription regulators (CRISPRa and CRISPRi), and targeted random mutation tools (Dual BEs, Target-G, EvolvR) (Figure 4). EvolvR and Target-G are efficient for the diversification of the

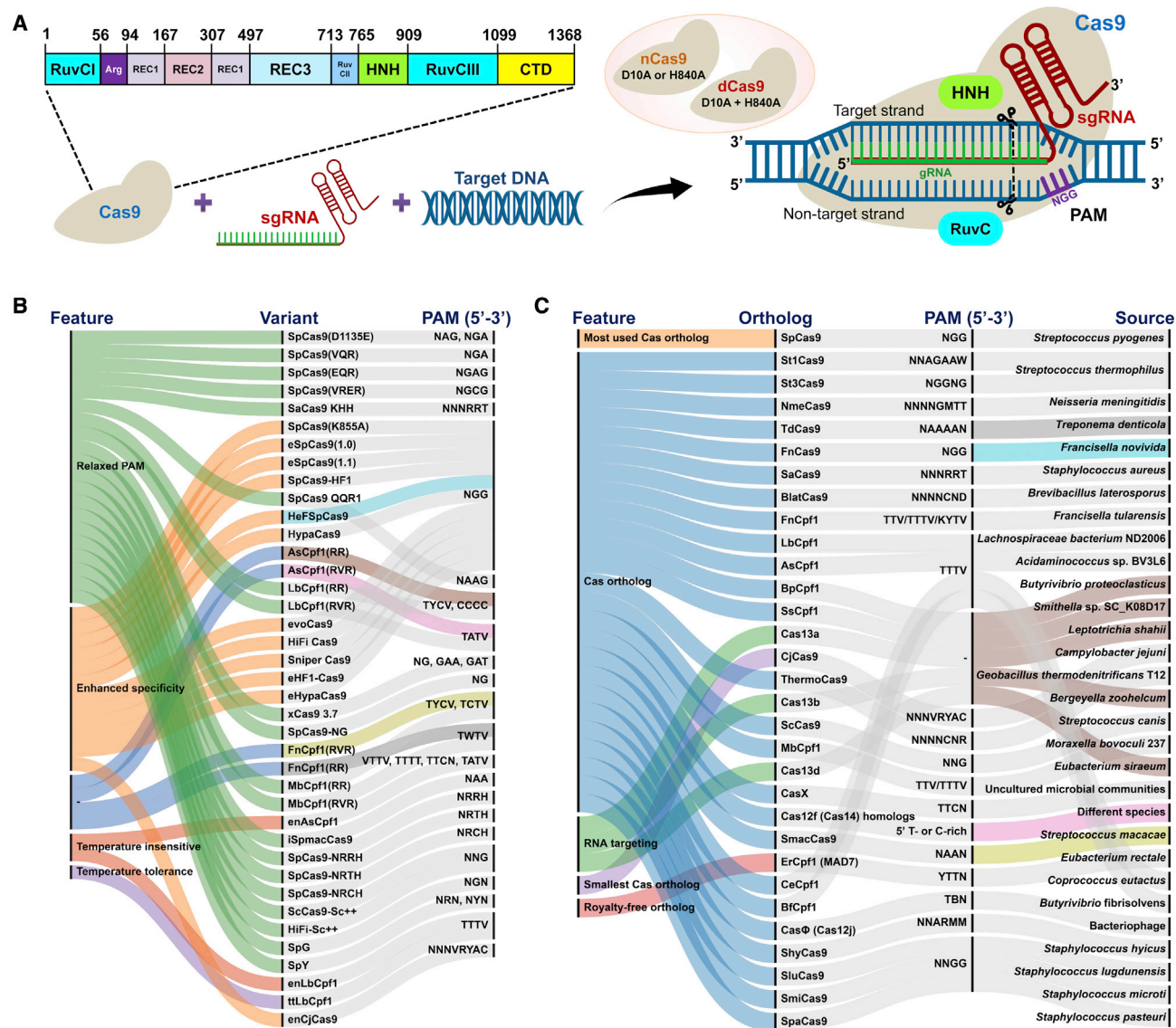


Figure 3. Basics of the CRISPR/Cas9 system, Cas variants, and orthologs.

(A) Mode of action of the CRISPR/Cas9 system is explained using SpCas9 as a model. The domain organization of the Cas9 enzyme consists of the recognition lobe (REC1, REC2, REC3), bridge helix (Arg), two nuclease domains (HNH, RuvC), and C-terminal protospacer adjacent motif (PAM) interacting domain (CTD). A ribonucleoprotein complex of the Cas9 enzyme (fully functional or impaired form, nickase or dead) with a single guide RNA (sgRNA) searches for the complementary target DNA region. Recognition of target DNA containing the PAM leads to structural rearrangements in Cas9, forming an R-loop in the Cas9-sgRNA-Target DNA complex. Two functional nuclease domains of wild-type Cas9 generate double-stranded breaks (DSBs) in the target DNA. The error-prone repair of DSBs produces insertions or deletions (indels), causing knockout of the encoded genes.

(B) Cas variants developed for CRISPR-based tool development.

(C) Cas orthologs used for CRISPR-based tool development. N is any nucleotide, R is A/G, M is A/C, D is A/G/T, B is G/T/C, W is A/T, V is G/C/A, R is A/G, and Y is C/T.

target locus in microbes, but their use in plants remains to be demonstrated.

Fusion of various effector molecules to partially impaired (Cas9 nickase, nCas9) or nuclease-deficient (dead Cas9, dCas9) Cas9 has been employed as a shuttle system to deliver the CRISPR-fused-cargo at the genomic site of interest. RNA-targeting Cas proteins enable several applications beyond simple RNA editing, such as nucleic acid detection, RNA degradation, RNA base editing, pathogen detection, and live RNA imaging, as summarized in

recently reviewed literature (Shelake et al., 2019a, 2019b; Pramanik et al., 2021). Plants cope with stress by several mechanisms that are governed by coding and non-coding regions in the plant genome and epigenetic marks as molecular signatures hidden in DNA packaging. The above-mentioned CRISPR-based tools can exploit various molecular mechanisms mediated by these genomic elements.

One of the significant applications of the CRISPR technique in plant molecular breeding is its capacity to produce chromosome

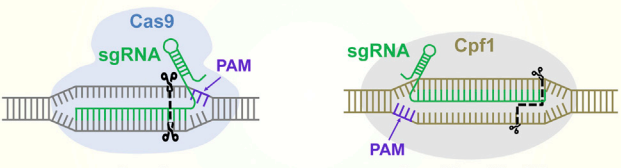
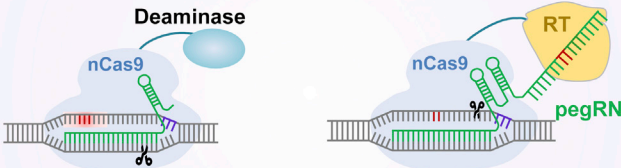
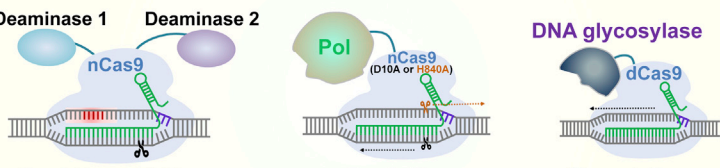
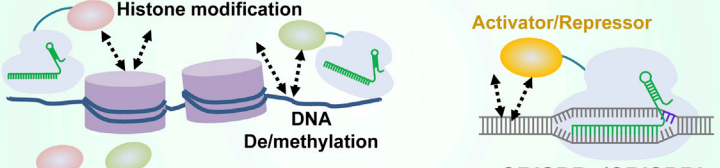

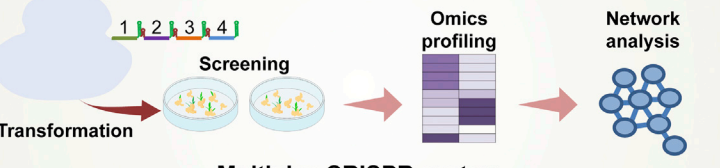
| CRISPR-based Tools | Applications |
|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
|  <p>Cas9 Cas12a (Cpf1)</p> | <p>DNA modification</p> <ul style="list-style-type: none"> Random Insertion/ Deletion/Substitution Gene targeting (GT) with donor template Targeted gene/promoter replacement Chromosomal rearrangements like inversion |
|  <p>Base editor Prime editor</p> | <p>Precision base editing</p> <ul style="list-style-type: none"> Cytosine BE (CBE) : C to T Adenine BE (ABE) : A to G C to Guanine BE (CGBE): C to G Prime Editor (PE): User-defined base change |
|  <p>Dual Base Editor EvolvR Target-G</p> | <p>Diversification of nucleotide bases in targeted window</p> <ul style="list-style-type: none"> Directed evolution of proteins Allele generation Promoter engineering |
|  <p>Epigenetic modifier CRISPRa/CRISPRi</p> | <p>Gene regulation</p> <ul style="list-style-type: none"> Epigenetic modifier: (de)methylation, (de)acetylation, chromatin (un)winding Transcription activation (CRISPRa) Transcription repression (CRISPRi) |
|  <p>RNA Cutting RNA Base Editing RNA Imaging</p> | <p>RNA targeting</p> <ul style="list-style-type: none"> RNA editing and degradation RNA Cytosine BE (CBE) : C to U RNA Adenine BE (ABE) : A to I/G RNA imaging |
|  <p>Multiplex CRISPR system</p> | <p>Multiplex CRISPR screening system</p> <ul style="list-style-type: none"> Simultaneous editing of multiple targets Genome-wide screening of gene families Metabolic pathway engineering Domain deletion in protein engineering |

Figure 4. CRISPR-based tools and their applications for plant GE.

CRISPR-based tools and their applications for DNA modification (indel generation), precise nucleotide substitution, random mutagenesis of a targeted region for novel allele generation, regulation of gene expression, RNA targeting, and CRISPR-based mutant library generation are summarized. Relevant information and references for Cas variants and orthologs are provided in the [supplemental information](#).

rearrangements, including inversion, translocation, duplication, or deletion, which enable the alteration of genetic linkages, generation, or disruption of open reading frames (ORFs) that govern critical agronomic traits (Huang and Puchta, 2021). CRISPR tools can induce different chromosomal rearrangements and produce stable GE lines in a single generation (Schmidt et al., 2020; Lu et al., 2021). For instance, programmed inversion of a 911-kb region on chromosome 1 and duplication of a 338-kb region on chromosome 2 facilitated a promoter swap between two genes and the creation of a novel gene cassette, conferring novel traits in rice (Lu et al.,

2021). Such precise editing at the chromosome level was considered an unrealistic task in the past. In this way, CRISPR tools are challenging several traditional notions, greatly expanding the potential of CRISPR tools for crop improvement.

APPLICATION OF CRISPR TOOLS FOR PLANT STRESS STUDIES

CRISPR-mediated plant studies related to abiotic stresses can be grouped into three clusters based on the type of target DNA

in the plant genome: structural genes, regulatory genes, and *cis*-regulatory elements (CREs) of structural or regulatory genes (Zafar et al., 2020). Structural genes play a pivotal role in enhancing plant stress tolerance. Regulatory genes encode proteins that contribute indirectly to stress tolerance, such as TFs, kinases, phosphatases, ion transporters, and miRNAs. CRISPR-mediated GE experiments may either stress susceptible or tolerant phenotypes, signifying the function of the target gene as a positive or negative regulator of a specific stress, respectively. In the following sections, we summarize recent CRISPR-based plant GE studies that have helped to reveal the underlying mechanisms involved in drought or salinity (Table 1) and their potential use in the design of stress-resilient crop varieties.

Exploring drought stress mechanisms using CRISPR tools

Drought directly restricts usable water supply for plants and thus has the most drastic growth-limiting effects. Owing to the polygenic nature of drought tolerance mechanisms, various genetic factors have been studied to understand their roles in drought response or to obtain drought-tolerant phenotypes. A range of strategies have been explored, including drought avoidance (stomatal closure, leaf rolling), TF editing (single or multiple copies in diploid or polyploid genomes), altering the expression of various genes (by epigenetic modification, promoter engineering, or miRNA editing), and modifying phytohormone signaling or catabolism pathways. Recent CRISPR-mediated drought studies that describe one of these strategies and their outcomes are summarized in the following paragraphs.

The early responses of plants to drought include ROS production and ABA-induced stomatal closure; these responses elicit long-distance signaling and help plants to cope with water loss and reduce water demand, respectively. PM-localized H⁺-ATPases generate proton gradients, energizing secondary transporters to bring ions and metabolites inside the cell (Palmgren, 2001). CRISPR/SpCas9 was successfully used to create novel alleles of *OST2* (previously *AHA1*) in *Arabidopsis*, and the resulting GE lines displayed enhanced response by altered stomatal closure under drought stress (Osakabe et al., 2016). Promotion of adaxial leaf rolling by increasing bulliform cell number is a critical morphological trait for drought avoidance. A leaf-rolling phenotype was obtained by knockout (KO) of *SRL1* and *SRL2*, which encode putative glycosylphosphatidylinositol (GPI)-anchored proteins (Liao et al., 2019). Homozygous GE lines exhibited higher production of antioxidant enzymes that reduced ROS activity, as well as better yield performance than the wild type.

TFs have been crucial candidates for understanding plant stress tolerance mechanisms. In particular, TFs from the previously mentioned families stimulate or suppress the transcription of many genes, imparting stress tolerance by modulating developmental and biochemical pathways (Nawkar et al., 2018; Gong et al., 2020). Thus, the CREs that determine the functions of TFs can reveal the fundamental mechanisms of quantitative traits and may assist in employing them for crop improvement. For instance, *NPR1*, a master regulator of plant defense response in tomatoes, was successfully inactivated by the CRISPR tool, and the resulting plants showed significant changes

in drought responses (Li et al., 2019). The *slnpr1* mutants displayed greater stomatal opening, more oxidative stress, and reduced antioxidant potential. Furthermore, the expression of several drought-responsive genes (*GST*, *DREB*) was severely affected, implying that SINPR1 has a positive role in tomato drought tolerance. In *Populus*, CRISPR-based studies of *PdNF-YB21* (Zhou et al., 2020) and *GNC* (Shen et al., 2021) were performed to understand the functions of these TFs in drought-related processes. Similarly, targeting of drought-responsive TFs such as *NAC14* from the NAC family (Liao et al., 2019) and *ERF83* from the AP2/ERF family (Jung et al., 2021) in rice using CRISPR tools produced drought-sensitive phenotypes, suggesting that both genes are positive regulators of drought-stress tolerance. Another CRISPR study revealed the pivotal roles of the antioxidant enzyme glutathione peroxidase and bZIP68 (GPX1-bZIP68) in redox sensing that activated osmotic stress-triggered signaling in rice (Zhou et al., 2021). The GPX1-bZIP68 module was found to confer higher osmotic or drought tolerance via an ABA-independent stress-response pathway.

Genetic engineering of polyploid plant genomes is complicated because of their complex genomic organization. In this regard, CRISPR tools could provide an ideal platform for polyploid crop GE because of their unique capabilities, like higher precision and multiplex editing. Kim and coworkers examined the effectiveness of CRISPR-based mutagenesis of two drought-related TFs (*DREB2* and *ERF3*) in protoplasts of polyploid bread wheat (Kim et al., 2018). Editing both of the targeted genes demonstrated the vast potential of CRISPR tools for the manipulation of polyploid plant genomes. A recent study characterized a new member of the NAC family, *NAC071-A* (Mao et al., 2022). Using the dual-sgRNA approach, deletion of two MYB promoter CREs (CAGTTA and TAACTG) confirmed *NAC071-A* as a positive regulator of drought stress tolerance in wheat. In addition, CRISPR-generated KO of *MYBL1* validated its role as an MYB transcriptional activator of *NAC071-A* expression, improving drought tolerance.

Epigenetic regulatory mechanisms, including chromatin remodeling, DNA/RNA methylation, and histone modification, play essential roles in response to environmental changes (Chang et al., 2020). Until recently, the molecular details of how epigenetic modifications coordinate stress-response mechanisms were elusive. AREB1 is a well-known positive regulator in the ABA signaling pathway and stress response in *Arabidopsis*, rice, and soybean (Oh et al., 2005; Yoshida et al., 2010; Barbosa et al., 2013). The histone acetyltransferase (HAT) enzyme relaxes chromatin folding and promotes enhanced gene expression. CRISPR activation (CRISPRa) and epigenetic modification approaches were combined to design a dCas9-HAT fusion system that upregulated *AREB1* expression, thereby boosting drought tolerance (Roca Paixão et al., 2019). GE lines exhibited stunted growth but higher chlorophyll content and rapid stomatal dynamics under drought stress. This is a classic example of epigenetic modification for improved drought tolerance. The enhanced expression of *AVP1* is another example of CRISPRa potential for the development of drought-tolerant plants (Park et al., 2017). *AVP1* has been implicated in auxin transport and proton pumping across the tonoplast, which indirectly enhances the activity of PM-localized H⁺-ATPases. Up to a five-fold increase in *AVP1* expression was reported, leading to

| Plant | Target | Type | Protein/Function | Editing outcome | Stress response | Reference |
|--------------------|-------------------------------------|-----------------|----------------------------------------------------------------|-------------------------------------------------|-----------------|---------------------------------|
| Drought | | | | | | |
| <i>Arabidopsis</i> | <i>OST2</i> | SG | PM proton ATPase | new alleles (insertion, deletion) | tolerance | (Osakabe et al., 2016) |
| <i>Arabidopsis</i> | <i>miR169a</i> | RG, miR | involved in adaptation to multiple stresses | KO (934 bp deletion) | tolerance | (Zhao et al., 2016) |
| <i>Arabidopsis</i> | <i>AVP1</i> | RG, phosphatase | vacuolar H ⁺ -pyrophosphatase | CRISPRa (transcription activation) | tolerance | (Park et al., 2017) |
| <i>Arabidopsis</i> | <i>AREB1</i> | RG, TF | ABA-responsive TF | epigenetic modification (chromatin unwinding) | tolerance | (Roca Paixão et al., 2019) |
| <i>Arabidopsis</i> | <i>TRE1</i> | RG | trehalase hydrolyzes trehalose into two molecules of D-glucose | KO (insertion, deletion) | tolerance | (Nuñez-Muñoz et al., 2021) |
| <i>Arabidopsis</i> | <i>STL1</i> | SG | dirigent protein localized to the Casparian strip | KO (deletion) | tolerance | (Wang et al., 2022) |
| Maize | <i>ARGOS8</i> | RG, promoter | negative regulator of ethylene response | swapping native promoter with GOS2 promoter | tolerance | (Shi et al., 2017) |
| Maize | <i>abh2</i> | RG | abscisic acid 8'-hydroxylase mediates stomatal opening | KO (insertion, deletion) | tolerance | (Liu et al., 2020c) |
| <i>Populus</i> | <i>NF-YB21</i> | RG, TF | positively regulates root growth | KO (insertion, deletion) | sensitive | (Zhou et al., 2020) |
| <i>Populus</i> | <i>GNC</i> | RG, TF | mediates stomatal closure | KO (insertion, deletion) | sensitive | (Shen et al., 2021) |
| Potato | <i>FLORE</i> | RG, lncRNA | counterpart of <i>StCDF1</i> (cycling DOF factor) | promoter knockdown (deletion of 770 and 952 bp) | sensitive | (Ramírez Gonzales et al., 2021) |
| Rapeseed | <i>A6.RGA</i> | RG | DELLA protein, negative regulator of GA signaling | KO (insertion, deletion) | tolerance | (Wu et al., 2020) |
| Rice | <i>DERF1, PMS3, MSH1, MYB5, SPP</i> | RG | genes related to amino acid synthesis and drought tolerance | KO (insertion, deletion) | tolerance | (Zhang et al., 2014) |
| Rice | <i>SAPK2</i> | RG, kinase | osmotic stress/ ABA-activated protein kinase | KO (deletion) | sensitive | (Lou et al., 2017) |
| Rice | <i>NAC14</i> | RG, TF | involved in growth and developmental changes | KO (deletion) | sensitive | (Shim et al., 2018) |
| Rice | <i>SRL1, SRL2</i> | SG | regulate leaf morphology | KO (deletion) | tolerance | (Liao et al., 2019) |
| Rice | <i>ERA1</i> | RG | regulates ABA signaling and the dehydration response | KO (insertion) | tolerance | (Ogata et al., 2020) |

Table 1. CRISPR-based drought and salinity stress-related GE studies in plants.

(Continued on next page)

| Plant | Target | Type | Protein/Function | Editing outcome | Stress response | Reference |
|--------------------|---------------------------|------------|-----------------------------------------------------------------------------------------|------------------------------------------------------------------|-----------------|------------------------------|
| Rice | <i>ERF83</i> | RG, TF | regulates drought response genes | KO (insertion) | sensitive | (Jung et al., 2021) |
| Rice | <i>GPX1</i> | RG | redox sensor and transducer | KO (deletion) | sensitive | (Zhou et al., 2021) |
| Rice | <i>bZIP68</i> | RG, TF | involved in ABA-independent osmotic stress response | KO (deletion) | sensitive | (Zhou et al., 2021) |
| Rice | <i>SAP</i> | SG | senescence-associated protein (SAP) | KO (insertion, deletion) | sensitive | (Park et al., 2022) |
| Tomato | <i>MAPK3</i> | RG, Kinase | signaling molecule in stress response | KO (insertion, deletion, substitution) | sensitive | (Wang et al., 2017) |
| Tomato | <i>NPR1</i> | RG, TF | receptor of salicylic acid | KO (insertion, deletion, substitution) | sensitive | (Li et al., 2019) |
| Tomato | <i>GID1a</i> | RG | GA receptor | KO (insertion) | tolerance | (Illouz-Eliaz et al., 2020) |
| Tomato | <i>LBD40</i> | RG | involved in JA-mediated stress response | KO (insertion) | tolerance | (Liu et al., 2020a) |
| Wheat | <i>DREB2, DREB3, ERF3</i> | RG, TF | DREBs, ethylene responsive factor (ERF) | KO (insertion, deletion) | sensitive | (Kim et al., 2018) |
| Wheat | <i>NAC071-A</i> | RG, TF | involved in growth and developmental changes | promoter KO (disruption of MYB recognition sites CAGTTA, TAACTG) | sensitive | (Mao et al., 2022) |
| Wheat | <i>MYBL1</i> | RG, TF | involved in growth and developmental changes | KO (deletion) | sensitive | (Mao et al., 2022) |
| Salinity | | | | | | |
| <i>Arabidopsis</i> | <i>C/VIF1</i> | RG | regulates cell wall and vacuolar invertase activities | KO (insertion, deletion) | tolerance | (Yang et al., 2020) |
| <i>Arabidopsis</i> | <i>SAUR41</i> | RG | auxin response gene, modulates cell expansion, ion homeostasis | KO (insertion, deletion) | sensitive | (Qiu et al., 2020a, 2020b) |
| <i>Arabidopsis</i> | <i>ACQOS</i> | SG | encodes four nucleotide-binding leucine-rich repeat proteins involved in osmo-tolerance | KO (insertion, deletion) | sensitive | (Kim et al., 2021) |
| Barley | <i>ITPK1</i> | RG, kinase | involved in phosphate storage and abiotic-stress signaling | KO (insertion, deletion) | sensitive | (Vičko and Ohnoutková, 2020) |
| Barley | <i>HVP10</i> | SG | involved in Na ⁺ sequestration in the tonoplast | KO (insertion) | sensitive | (Fu et al., 2021) |

Table 1. Continued

(Continued on next page)

| Plant | Target | Type | Protein/Function | Editing outcome | Stress response | Reference |
|---------|---------------------|------------|----------------------------------------------------------------------------|----------------------------------------|-----------------|----------------------------------------|
| Cotton | <i>AITR</i> genes | RG, TF | DPA4 is involved in plant development, SOD7 regulates seed size | KO (deletion) | tolerance | (Wang et al., 2021a) |
| Maize | <i>CLCg</i> | SG, IT | involved in chloride transport | KO (insertion, deletion) | sensitive | (Luo et al., 2021) |
| Maize | <i>HKT1</i> | SG, IT | controls root-shoot Na ⁺ delivery | KO (deletion) | sensitive | (Zhang et al., 2018a) |
| Maize | <i>STL1</i> | SG | dirigent protein localized to the Casparian strip | KO (insertion, deletion) | tolerance | (Wang et al., 2022) |
| Pumpkin | <i>RBOHD</i> | SG | NADPH oxidase | KO (deletion) | sensitive | (Huang et al., 2019) |
| Rice | <i>RAV2</i> | RG, TF | functions in the regulation of developmental processes | promoter element deletion | tolerance | (Duan et al., 2016) |
| Rice | <i>MIR528</i> | RG, miR | regulates salinity stress through downregulation of salinity-related genes | KO (insertion, deletion, substitution) | sensitive | (Zhou et al., 2017) |
| Rice | <i>SAPK1, SAPK2</i> | RG, kinase | osmotic stress/ ABA-activated protein kinases | KO (insertion, deletion) | sensitive | (Lou et al., 2018) |
| Rice | <i>BBS1</i> | RG, kinase | receptor-like cytoplasmic kinase | KO (deletion of 581 bp) | sensitive | (Zeng et al., 2018) |
| Rice | <i>RR9, RR10</i> | RG | negatively regulate cytokinin signaling | KO (deletion) | tolerance | (Wang et al., 2019) |
| Rice | <i>DOF15</i> | RG, TF | regulates primary root elongation | KO (insertion, deletion) | sensitive | (Qin et al., 2019) |
| Rice | <i>SPL10</i> | RG, TF | regulates trichome development | KO (insertion, deletion) | tolerance | (Lan et al., 2019) |
| Rice | <i>RR22</i> | RG, TF | involved in cytokinin-dependent gene expression | KO (insertion, deletion, substitution) | tolerance | (Zhang et al., 2019; Han et al., 2022) |
| Rice | <i>NAC041</i> | RG, TF | involved in salt stress | KO (insertion, deletion) | sensitive | (Bo et al., 2019) |
| Rice | <i>NCA1a, NCA1b</i> | SG | chaperone protein modulates catalase activity | KO (insertion, deletion) | sensitive | (Liu et al., 2019) |
| Rice | <i>GTγ-2</i> | RG, TF | binds to GT-1 promoter element and regulates growth and development | KO (insertion) | sensitive | (Liu et al., 2020d) |
| Rice | <i>PIL14</i> | RG, TF | directly binds to the promoter of cell elongation-related genes | KO (deletion) | sensitive | (Mo et al., 2020) |
| Rice | <i>PQT3</i> | RG | E3 ubiquitin ligase | KO (insertion, deletion) | tolerance | (Alfatih et al., 2020) |

Table 1. Continued

(Continued on next page)

| Plant | Target | Type | Protein/Function | Editing outcome | Stress response | Reference |
|-----------------------------------------------------------------------------------------|------------------------------------------------------------------------|------------|--------------------------------------------------------------------------------|-------------------------------------------|---------------------------------------------------|-----------------------|
| Rice | <i>BG3</i> | RG | purine permease, cytokinin transport | KO (insertion, deletion) | sensitive | (Yin et al., 2020) |
| Rice | <i>FLN2</i> | RG, kinase | involved in sugar partitioning | KO (insertion) | sensitive | (Chen et al., 2020) |
| Rice | <i>SOS1</i> | SG, IT | Na ⁺ /H ⁺ antiporter mediating Na ⁺ transport | insertion of 60-bp translational enhancer | tolerance | (Lu et al., 2020) |
| Rice | <i>Gl</i> | RG | circadian clock component | KO (deletion) | tolerance | (Wang et al., 2021b) |
| Rice | three <i>ELF4</i> homologs | RG | circadian clock components | KO (deletion) | sensitive | (Wang et al., 2021b) |
| Rice | <i>bHLH024</i> | RG, TF | basic helix–loop–helix TF involved in growth and stress responses | KO (deletion) | tolerance | (Alam et al., 2022) |
| Soybean | <i>NHX5</i> | SG, IT | Na ⁺ /H ⁺ exchanger (NHX) transmembrane protein | KO (insertion, deletion) | sensitive | (Sun et al., 2021) |
| Tomato | <i>HAK20</i> | SG, IT | involved in K ⁺ and Na ⁺ homeostasis | KO (insertion, deletion) | sensitive | (Wang et al., 2020a) |
| Tomato | <i>HyPRP1</i> | RG | transmembrane protein involved in multiple stresses | domain deletion | tolerance | (Tran et al., 2021) |
| Tomato | <i>SOS1</i> | SG, IT | Na ⁺ /H ⁺ antiporter mediating Na ⁺ transport | KO (insertion, deletion) | sensitive | (Wang et al., 2021c) |
| Tomato | <i>ABIG1</i> | RG, TF | homeodomain-leucine zipper (HD-ZIP) TF | KO (deletion) | tolerance | (Ding et al., 2022) |
| Wheat | Two <i>HAG</i> homologs | RG | modulate ROS production and signaling | KO (insertion, deletion) | sensitive | (Zheng et al., 2021) |
| Multiple stresses (including drought, salinity, or both stresses simultaneously) | | | | | | |
| <i>Arabidopsis</i> | <i>UGT79B2</i> , <i>UGT79B</i> | SG | UDP-glycosyltransferases | KO (insertion) | sensitive to drought, salinity, cold | (Li et al., 2017) |
| <i>Arabidopsis</i> | <i>DPA4</i> , <i>SOD7</i> , <i>AITR2</i> , <i>AITR5</i> , <i>AITR6</i> | RG, TF | <i>DPA4</i> is involved in plant development, <i>SOD7</i> regulates seed size | KO (insertion, deletion) | increased seed size and drought tolerance | (Chen et al., 2019) |
| <i>Arabidopsis</i> | <i>ArathEULS3</i> | SG | <i>Euonymus europaeus</i> -related lectin | KO (insertion, deletion) | tolerance to osmotic stress, salinity | (Dubiel et al., 2020) |
| <i>Arabidopsis</i> | <i>AIW1</i> , <i>AIW2</i> | RG, TF | regulate multiple aspects of growth and development | KO (insertion, deletion) | sensitive to ABA but no difference in salt stress | (Wang et al., 2020b) |
| <i>Arabidopsis</i> | <i>AITR</i> family | RG, TF | ABA-induced transcriptional repressors | KO (insertion, deletion) | tolerance to drought, salinity | (Chen et al., 2021a) |
| <i>Arabidopsis</i> | <i>WRKY3</i> , <i>WRKY4</i> | RG, TF | TFs involved in stress responses | KO (deletion) | sensitive to drought, Me-JA stress | (Li et al., 2021) |

Table 1. Continued

(Continued on next page)

| Plant | Target | Type | Protein/Function | Editing outcome | Stress response | Reference |
|---------|---------------------------------------|---------|----------------------------------------------|--------------------------|---------------------------------------------------------------------------------|------------------------------|
| Rice | <i>DST</i> | RG, TF | zinc-finger TF | KO (insertion, deletion) | tolerance to drought, salinity | (Santosh Kumar et al., 2020) |
| Rice | <i>PQT3</i> | SG | E3 ubiquitin ligase | KO (insertion, deletion) | tolerance to salinity, oxidative stress | (Alfatih et al., 2020) |
| Rice | <i>NAC006</i> | RG, TF | involved in growth and developmental changes | KO (insertion, deletion) | sensitive to drought, heat | (Wang et al., 2020c) |
| Rice | <i>miR535</i> | RG, miR | involved in plant growth and development | KO (deletion) | tolerance to drought, salinity | (Yue et al., 2020) |
| Soybean | <i>MYB118</i> | RG, TF | MYB TF | KO (substitution) | sensitive to drought, salinity | (Du et al., 2018) |
| Tomato | <i>ARF4</i> | RG, TF | auxin response factor | KO (deletion) | tolerance to osmotic stress, salinity | (Bouzroud et al., 2020) |
| Tomato | <i>GRXS14, GRXS15, GRXS16, GRXS17</i> | SG | components of ROS scavenging network | KO (insertion, deletion) | sensitive to heat, chilling, drought, heavy metal toxicity, nutrient deficiency | (Kakeshpour et al., 2021) |

Table 1. Continued

SG, structural gene; RG, regulatory gene; TF, transcription factor; KO, knockout; CRISPRa, CRISPR activation system; miR, microRNA; lncRNA, long non-coding RNA; DOF, DNA-binding with one-finger TF family; IT, ion transporter.

improved performance under drought conditions. In the future, ABA pathway-related genes can be targeted using CRISPRa tools that provide novel avenues for the design and development of drought-response strategies.

Regulatory elements including intronic and 5' or 3' untranslated regions (UTRs) regulate desired gene expression levels. Promoter and CRE editing are attractive strategies for reprogramming transcription. For example, in maize, the native *ARGOS8* promoter was replaced with the constitutive *GOS2* promoter in the 5' UTR of *ARGOS8* using dual sgRNAs (Shi et al., 2017). *ARGOS8* is involved in the negative regulation of ethylene responses and interacts with ethylene response factors. CRISPR-generated *GOS* promoter-*ARGOS8* GE lines exhibited improved drought tolerance and increased crop yield.

Small and long non-coding RNAs (lncRNAs) have emerged as crucial GE targets for crop improvement. miRNAs have been found to control the expression of target mRNAs by providing instructions to repress post-transcriptional expression or direct degradation (Zhang, 2015). In *Arabidopsis*, simultaneous deletion and gene replacement using a novel combinatory dual-sgRNA/SpCas9 vector generated *mir169a* mutants that displayed enhanced drought tolerance phenotypes (Zhao et al., 2016). The CDF TF family members control water homeostasis and drought stress conditions (Corrales et al., 2017). A recent study in potato investigated the combined role of CDF1 and its lncRNA counterpart gene *FLORE* by CRISPR targeting of promoter motifs in the *FLORE* locus (Ramírez Gonzales et al., 2021). Exposure of GE lines to drought stress demonstrated that CDF1 regulates *FLORE* expression by binding to its promoter motifs, whereas *FLORE* acts as a natural antisense

transcript that regulates *CDF1* transcription. Therefore, miRNAs or lncRNAs can be targeted by CRISPR tools to understand their roles in stress tolerance.

ABA signaling components such as *ERA1*, which encodes the β -subunit of farnesyltransferase, regulate the dehydration response in *Arabidopsis* and rice. CRISPR-edited *ERA1* mutant lines displayed enhanced tolerance to drought stress (Ogata et al., 2020). In tomato, CRISPR-mediated study of the GA receptor *GID1a* showed that *GID1a* contributes to improved water-holding capacity and stomatal conductance (Illouz-Eliaz et al., 2020). The CRISPR-generated *gid1* mutants displayed normal growth with decreased transpiration rates and improved recovery from transient water stress without compromising crop yield. The rapeseed genome contains four *RGA* genes involved in synthesizing DELLA proteins, which inhibit GA signaling. The KO mutant of *A6.RGA* showed enhanced drought tolerance, confirming this gene as a negative regulator of drought stress-related GA signaling (Wu et al., 2020). CRISPR-derived mutant lines of *LBD40* shed light on previously unknown functional aspects of the LBD family (Liu et al., 2020a). The SILBD40 protein participates in JA signaling and confers improved drought tolerance. In microbes and plants, non-reducing trehalose functions as an osmoprotectant against osmotic stress. Through genetic engineering, enhanced trehalose biosynthesis in rice resulted in tolerance to drought, salinity, and soil sodicity (Joshi et al., 2020b). Therefore, modifying the catabolic pathway of trehalose by targeting trehalase (the enzyme that hydrolyzes trehalose) is an attractive way to develop drought-tolerant GE lines. *Arabidopsis* GE mutants for *TRE1*, which encodes the trehalase enzyme, exhibited enhanced drought tolerance (Nuñez-Muñoz et al., 2021).

Exploring plant responses to salinity stress using CRISPR tools

Higher salt concentrations pose a considerable threat to plant growth and development by impeding physiological processes through osmotic stress, nutrient imbalance, ionic toxicity, and oxidative stress (Shrivastava and Kumar, 2015). A combination of biochemical, physiological, and molecular processes regulates available salt concentrations by adjusting ionic tolerance, tissue-specific tolerance, and osmotic tolerance. CRISPR targeting of TFs is a general approach for gaining molecular insights into their regulatory roles in salt-response pathways. KO of salt-stress-related proteins, including TFs, causes either improved stress tolerance or higher susceptibility, depending on their effects on downstream regulatory pathways. In this section, CRISPR-mediated salt-related studies are discussed by considering the targeted genes as positive (sensitive) or negative (tolerance) regulators, depending on the KO effect on plant phenotype.

Genetic factors that contribute to improved stress tolerance are considered to be positive regulators. For instance, *RAV2* encodes a TF from the AP2/ERF family in rice. CRISPR-mediated deletion of GT-1 (GAAAAA), a promoter element in the *RAV2* locus, confirmed its involvement in salt response (Duan et al., 2016). The GT-1 element was identified as a regulatory region, given that its deletion eliminated salt-responsive *RAV2* expression. Several TFs act as positive salt-stress-responsive factors, and salt-sensitive phenotypes were observed in GE lines of *DOF15* (Qin et al., 2019), *NAC041* (Bo et al., 2019), *GT-2* (Liu et al., 2020d), and *PIL14* (Mo et al., 2020). Examples of CRISPR-mediated targeting of kinase and phosphatase genes include *FLN2* in rice (Chen et al., 2020), *BBS1* in rice (Zeng et al., 2018), and *ITPK1* in barley (Vičko and Ohnoutková, 2020); the edited plants showed salt-responsive phenotypes with reduced tolerance. Apart from TFs or kinases, other salt-stress-responsive factors have been studied using CRISPR tools; examples include *MIR528* in rice (Zhou et al., 2017), *RBOHD* in pumpkin (Huang et al., 2019), *NCA1a/OsNCA1b* in rice (Liu et al., 2019), *SAUR41* in *Arabidopsis* (Qiu et al., 2020a, 2020b), the *ACQOS* gene cluster in *Arabidopsis* (Kim et al., 2021), *BG3* in rice (Yin et al., 2020), three *ELF4* homologs in rice (Wang et al., 2021b), *HAG1* in hexaploid wheat (Zheng et al., 2021), and *HVP10* in barley (Fu et al., 2021).

Some genes function as negative regulators of plant response to salt and other abiotic stresses. *RR22*, which encodes a type-B response regulator involved in cytokinin signaling, was knocked down using CRISPR/Cas9, thereby improving rice salt tolerance (Zhang et al., 2019). Transgene-free homozygous GE lines were obtained by T1 segregation, which is likely to offer the possibility of trait introgression in related varieties within a short time. The CRISPR-derived *SPL10* mutant, a member of the SPL family, displayed a salt-tolerant phenotype, suggesting that OsSPL10 is a negative regulator of rice response to salt stress (Lan et al., 2019). In addition, *SPL10* was found to positively regulate trichome formation. Another study reported improved agronomic performance under drought and salt stress by novel base-insertion mutants of *DST*, which encodes a zinc-finger TF, in indica rice cv. MTU1010 (Santosh Kumar et al., 2020). Additional examples of negative regulators studied with CRISPR tools include *C/VIF1* in rice, encoding a proteinaceous

inhibitor of fructosidases involved in ABA-mediated responses (Yang et al., 2020); *HyPRP1* in tomato, encoding a hybrid proline-rich cell wall-embedded structural protein (Tran et al., 2021); *PQT3* in rice, encoding an E3 ubiquitin ligase involved in oxidative and salt stress (Alfatih et al., 2020); *RR9* and *RR10* in rice, encoding type-A RR proteins implicated in cytokinin signaling (Wang et al., 2019); and a rice homolog of *GI*, an evening-phased circadian clock component (Wang et al., 2021b).

Transporter proteins facilitate the trafficking of ions and other molecules over short (cell to cell) or long distances (across tissues and organs) in the plant body (Deshmukh et al., 2021). Plants maintain appropriate Na^+/K^+ balance via ion transporters to endure salt stress (Figure 3). In this regard, plant ion transporters are critical to understanding plant salt-stress response mechanisms. Na^+ transporter families include ENA, NHX, HKT, ATPases, and cation exchangers, and K^+ transporter families consist of voltage-gated channels/shakers, the KT/HAK/KUP family, VGICs, and KCO channels (Tan et al., 2022). Using CRISPR-mediated mutagenesis, frameshift (5-bp deletion) and truncation (34-bp deletion) mutants of *HKT1* were obtained (Zhang et al., 2018a) to characterize the function of the *HKT1* locus in the polyploid maize genome. Reduced salt tolerance in GE lines confirmed *HKT1* as a positive regulator that promotes Na^+ removal from the leaf by transporting Na^+ from xylem sap (root to shoot). Similarly, functional aspects of the ion transporters *NHX5* (Sun et al., 2021) and *CLCg* (Luo et al., 2021) were examined using CRISPR tools in the salt responses of polyploid soybean and maize species. CRISPR screening of mutants for *HAK20* (Wang et al., 2020a) and *SOS1* (Wang et al., 2021c) confirmed that genetic variations in these loci also mediate phenotypic changes associated with salinity tolerance in tomato crops. Notably, disruption of the CRT/DRE promoter element (CCGAC) recognized by the CBF/DREB family of TFs produced a susceptible phenotype under salt stress.

Exploring multiple stress responses with CRISPR tools

CRISPR-based multi-stress plant studies, including those on drought or salinity, are summarized in this section (Table 1). Phytohormones such as ABA, GA, and cytokinins play a significant role in tolerance to multiple biotic and abiotic stresses. Thus, genes related to phytohormone biosynthesis and signaling usually influence plant responses to various stresses. For instance, *SAPK1* and *SAPK2* enzymes from the SnRK2 family mediate ABA signaling in rice. Loss-of-function *sapk2* mutants produced by CRISPR/Cas9 were insensitive to ABA (Lou et al., 2017). The *sapk2* mutants displayed high sensitivity to dehydration and ROS, highlighting the role of *SAPK2* in drought stress. Later, double-mutant (*sapk1/sapk2*) lines were exposed to osmotic and salt stresses and exhibited sensitive phenotypes (Lou et al., 2018). These observations indicate that *SAPK1* and *SAPK2* together act as positive regulators of tolerance to osmotic and salt stress.

Kinases from the MAPK family play crucial roles in plant responses to biotic and abiotic stresses. Protein kinases add phosphate groups to substrate proteins, thereby regulating their functions during various cellular processes (Wang et al., 2020d). A CRISPR-based approach was used to reveal functional aspects of the *MAPK3* enzyme in tomato. KO of *MAPK3* led to a

drought-sensitive phenotype (Wang et al., 2017) and also decreased resistance to the necrotrophic pathogen *Botrytis cinerea* (Zhang et al., 2018b), suggesting that MAPK3 is a positive regulator of drought tolerance and pathogen resistance. By contrast, *MAPK3* mutants showed enhanced thermotolerance compared with WT plants, indicating that MAPK3 is a negative regulator of heat stress tolerance (Yu et al., 2019). It is therefore crucial to consider the trade-off between distinct stress responses and cellular functions while targeting genes involved in multiple cellular processes.

Gene expression and analysis in KO lines of *MYB118*, which encodes a nuclear-localized TF, revealed its involvement in plant responses to drought, salt, and high temperature. GE plants exhibited reduced tolerance to these stresses, indicating a positive role for *MYB118* in the maintenance of osmotic homeostasis and cellular oxidation status (Du et al., 2018). Some more examples of multi-functional TFs targeted by CRISPR include *ARF4*, improving tolerance to salinity and osmotic stress in tomato (Bouzroud et al., 2020); *NAC006* in rice, reducing drought and heat tolerance (Wang et al., 2020c); *AIW1* and *AIW2* in *Arabidopsis*, conferring hypersensitivity to ABA but having no effect on salt-stress tolerance (Wang et al., 2020b); six *AITR* (1, 2, 3, 4, 5, and 6) genes in *Arabidopsis*, resulting in enhanced drought and salt tolerance (Chen et al., 2021a); and *WRKY3* and *WRKY4* in *Arabidopsis*, reducing tolerance to salt stress and methyl jasmonate (Me-JA) treatment (Li et al., 2021). The *OsmiR535* gene from the highly conserved miRNA family is a well-known regulator of plant thermotolerance. CRISPR-derived *miR535* loss-of-function mutants performed better under drought, salt, high ABA, and osmotic stresses (Yue et al., 2020). *Arabidopsis* quintuple-mutant lines (*AITR2*, *AITR5*, *AITR6*, *DPA4*, and *SOD7*) were generated by multiplex GE and showed increased seed size and drought tolerance (Chen et al., 2019).

Regulation of ROS moieties is an alternative strategy employed by plants to overcome abiotic stresses. Glutaredoxins (GRXs) from the thioredoxin superfamily function as ROS scavengers. Multiplex editing of four GRX genes (*GRXS14*, *GRXS15*, *GRXS16*, and *GRXS17*) in tomato produced a combination of double, triple, and quadruple mutants that exhibited hypersensitive phenotypes under abiotic stresses like drought, heat, heavy metal toxicity, nutrient deficiency, chilling, and light (Kakeshpour et al., 2021). Another study demonstrating the effective use of the multiplex editing approach included double-KO lines (*ugt79b2/ugt79b*) of *UGT* homologs in *Arabidopsis* (Li et al., 2017). These genes encode glycosyltransferase enzymes involved in the anthocyanin biosynthesis pathway and abiotic-stress responses. Double-mutant lines were hypersensitive to various abiotic stresses, demonstrating the crucial functions of *UGT79B2* and *UGT79B* in anthocyanin production and subsequent stress tolerance. Negative (instead of positive) regulators of plant stress responses would be ideal CRISPR targets for future development of multi-stress-tolerant crops.

INTEGRATING MODERN BREEDING APPROACHES WITH CRISPR TOOLS

The main goal of researchers in exploring plant stress pathways is to develop stress-resilient varieties by modulating the relevant

genomic aspects of the desired crop species. Despite recent progress in understanding genetic factors linked to plant stress, there is still a need to gain more insights into the basis of genetic variations responsible for contrasting phenotypes under drought or salinity stress in related varieties of the same crop species. Alternatively, orthologs of the same gene may govern different traits in related plant species, thus requiring molecular characterization. This section evaluates the applications of CRISPR technology and GAB approaches for the development of plant drought/salinity stress tolerance (Figure 5).

Finding regulatory genetic factors of drought/salinity stress in plants is a major challenge. Several domesticated plant species have lost the genetic variations responsible for quantitative traits during selective breeding, although these traits still reside in wild relatives (Wang et al., 2020a). Alternatively, domesticated species acquire a set of new heritable variations linked to improved fitness under stress conditions (Groen et al., 2022). With advances in genome sequencing methods, high-quality whole-genome sequences of domesticated species and their wild relatives are available in the public domain (Pazhamala et al., 2021). Genomic comparisons between cultivated and wild species may facilitate the identification of these genetic factors that can be introduced into commercial cultivars using CRISPR tools. Through CRISPR engineering, wild relatives that are naturally tolerant to drought/salinity stress can be domesticated by inserting mutations found in the gene pool of the cultivated species, a method described as *de novo* domestication. This approach enables the preservation of genetic diversity, as shown by recent studies in tomato (Zsögön et al., 2018) and rice (Yu et al., 2021a, 2021b).

The availability of whole-genome sequences for important crops has also facilitated the identification of large gene families, members of which are known to play important roles in plant stress tolerance (Kushwaha et al., 2009; Arya et al., 2014; Singh et al., 2015b; Bhuria et al., 2019). However, determining the exact function of each family member is a challenging task. To this end, precision GE tools have been used to develop large KO mutant libraries that enable easier identification of causal genes with greater specificity (Lu et al., 2017; Liu et al., 2020b). The function of LRR-XII family genes was investigated by developing a CRISPR library in tomato (Jacobs et al., 2017). In a single transformation with three sgRNAs per gene, 54 genes were targeted, resulting in 31 GE plants.

Similarly, targeted mutagenesis of an entire gene family may be achieved in order to functionally characterize all of its members. Moreover, a high-throughput approach for genome-scale mutagenesis of rice genes was developed using an sgRNA library, and more than 91 000 targeted loss-of-function mutants were generated (Lu et al., 2017). This pooled CRISPR library approach for genome-wide mutagenesis also enables the development of mutant libraries in non-model plant species. Recently, quantitative trait loci (QTL)-based mapping, evolutionary systems biology, genome-wide association studies (GWAS), and haplotype-based breeding have proven helpful in the discovery of polygenic adaptations that distinguish stress-prone and resistant cultivars. Among the significant genetic targets associated with plant stress are the CREs, which can drive dose-dependent gene expression, wild species domestication,

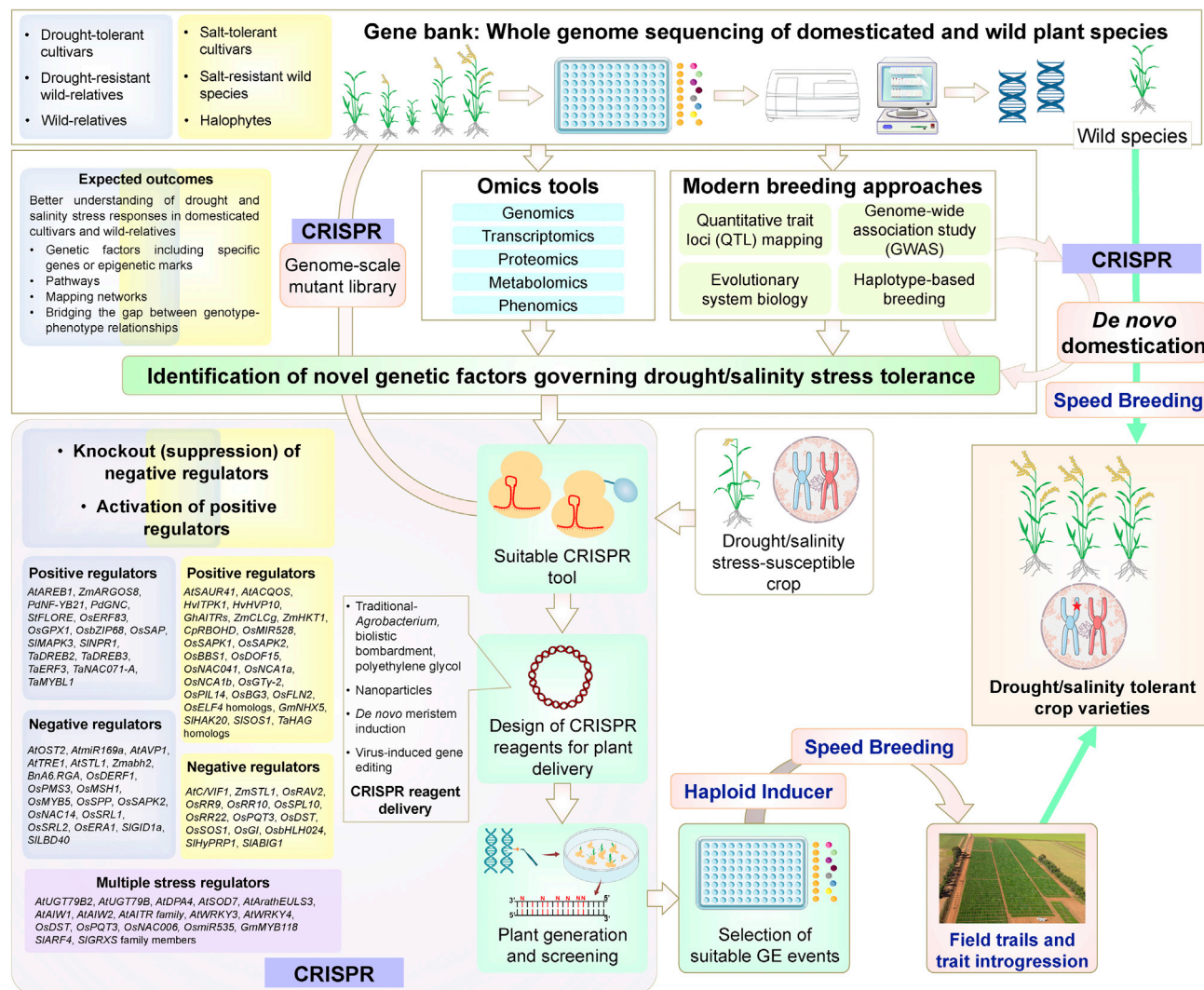


Figure 5. Overview of the integration of genomic-assisted breeding and other approaches with CRISPR technology to develop drought/salinity-tolerant crops.

Known positive and negative regulators of drought (blue box) and salinity (yellow box) stress tolerance are ideal targets for crop improvement. Descriptions of different aspects depicted here are provided in the main text.

and breeding innovation (Swinnen et al., 2016; Springer et al., 2019). Integration of CRISPR tools with some novel approaches shows excellent potential for expediting the rate of crop improvement; for instance, through speed breeding (Watson et al., 2018), *de novo* meristem induction (Maher et al., 2020), and nanomaterial-mediated delivery (Demirer et al., 2021). In particular, speed breeding (with controlled light and temperature conditions) can accelerate the introgression of improved drought/salinity tolerance traits into elite cultivars by either GAB approaches or rapid screening of CRISPR-generated GE lines.

Introgression of drought or salinity tolerance traits into elite cultivars by crossing with haploid inducer (HI) lines is highly desirable. HI lines generate viable plants that contain a half set of chromosomes, and chromosome doubling produces homozygous double HI plants. CRISPR-generated *in vivo* HI lines ideally serve this purpose, enabling one-step trait introgression

into elite crop varieties without the loss of commercial traits during genetic segregation (Kelliher et al., 2019; Khanday et al., 2019).

PERSPECTIVES AND CHALLENGES

Molecular information on the functions of multiple genes and the interactions among members of different protein families during plant stress is limited, posing challenges for the selection of suitable GE targets. Hence, functional validation of stress-governing genes and protein families in different crops is required. In the following section, we discuss various aspects of drought/salinity stress that would enable the engineering of stress-tolerant crops in the future. These aspects include exploration of genetic factors related to drought/salinity stress, control of gene expression, engineering of multiple pathways, manipulation of natural stress-tolerant genetic materials, and strategies to exclude toxic molecules from the cytosol. Some of these approaches are depicted

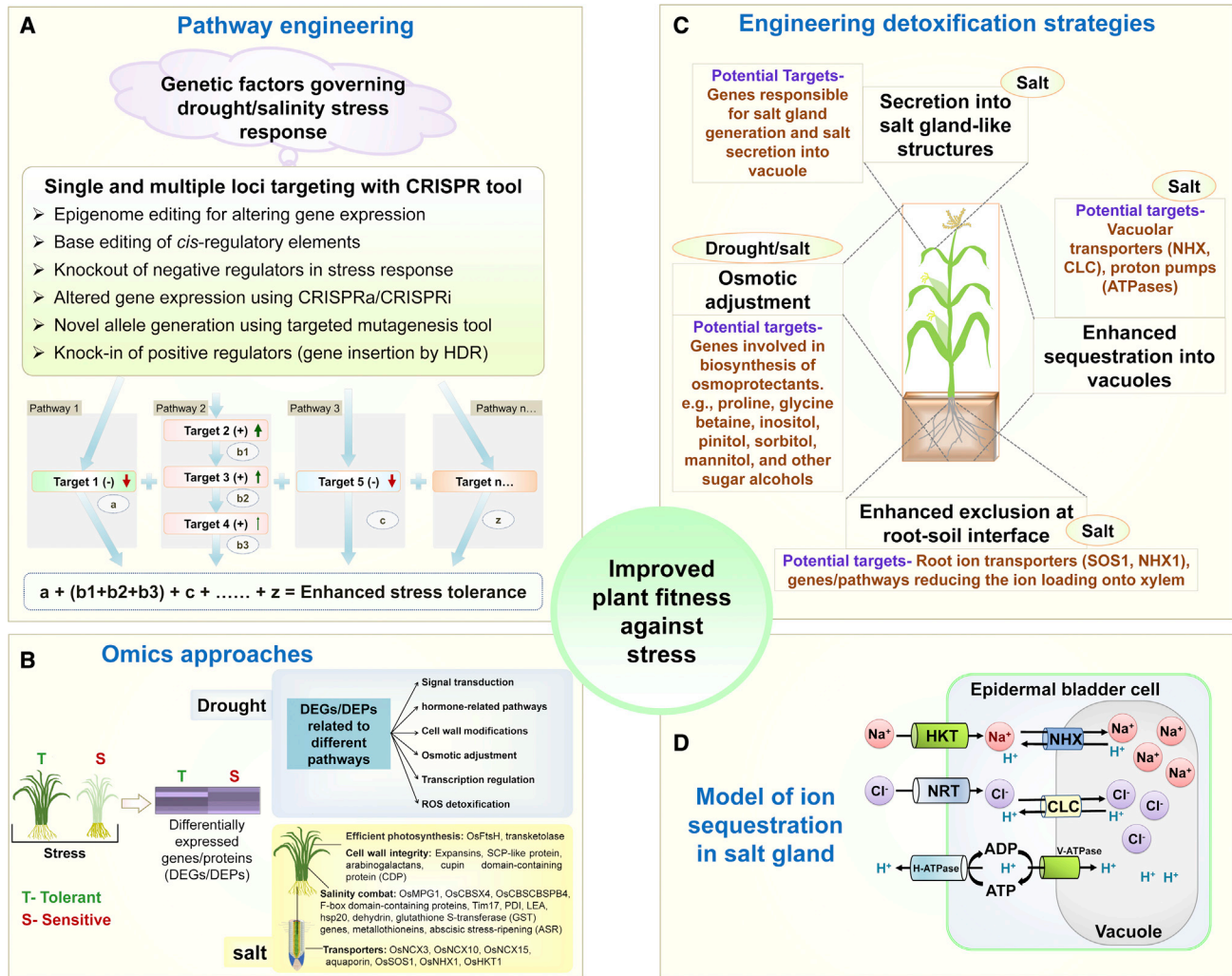


Figure 6. Strategies proposed for the engineering of plant tolerance to drought and salinity stress using CRISPR-based tools. (A) Single or multiple genetic elements can be targeted using desired CRISPR tools. Genes from different pathways responsible for drought or salinity tolerance in plants can be either knocked out (negative element: target 1 or 5, red arrow) or knocked in (positive element: target 2, green arrow). In hypothetical pathway 2, the thickness of the green arrows depicts the major or minor role of the edited target gene in the stress condition. Epigenetic modifiers and CRISPR activation/interference (CRISPRa/CRISPRi) tools provide another means of regulating gene expression without introducing any mutations. In addition, base substitution tools such as base editors enable editing of *cis*-regulatory elements or generation of novel alleles. Targeting a single gene from a specific pathway may not be sufficient for developing stress tolerance in the case of polygenic traits like drought and salinity. Therefore, targeting of multiple genes from functionally related pathways would be ideal for achieving improved drought and salinity stress tolerance. (B) Differentially expressed genes found in comparative omics analyses of stress-tolerant and sensitive cultivars are ideal candidates for GE to enable further characterization and exploitation in modern breeding. Details are provided in the supplemental information. (C) Detoxification strategies are described in the main text. (D) In the salt gland model, HKT1 transports Na⁺ and NRT transports Cl⁻ to the gland cell; i.e., the epidermal bladder cell. NHX1 and CLC sequester excess Na⁺ and Cl⁻ in the vacuole. H-ATPase and V-ATPase generate proton gradients to drive ion transport.

in Figure 6. Finally, we briefly discuss potential areas for improvement of CRISPR applications in plants.

Exploring the basics of stress-responsive genetic factors

Fragmented information on stress sensors, early signaling mechanisms, and regulation of gene expression are significant challenges for understanding the intricacies of abiotic-stress response and tolerance mechanisms. Our understanding of the connecting links between identified or unknown sensors, early

signaling processes, and crosstalk among different pathways is still incomplete. Another challenging aspect being dealt with by researchers is the fact that various protein family members exhibit redundant functions in stress-response pathways (Van Zelm et al., 2020). Moreover, plant scientists aim to understand how members of various gene families, like protein kinases, TFs, and ion transporters, influence each other's functions during encounters with single or multiple stresses. Thus, further characterization of different gene family members in commercial crops would be the starting point for exploring the roles of stress-responsive factors in plant life and for their

subsequent use in the design of stress-resilient varieties for the future. CRISPR-mediated targeted mutagenesis of genes with unknown or redundant functions may help to characterize their roles in plant stress biology, as evidenced by the newly identified AITR family (Chen et al., 2019, 2021a; Wang et al., 2021a).

Epigenetic regulation and related mechanisms are central to the fate of gene expression and priming or memory development (Agarwal et al., 2020). Chromatin structure dictates the underlying gene expression, presumed to be active, dead, or repressed in various situations. Information about epigenetic changes and their transfer from one generation to the next is essential for realizing the whole scenario of stress memory development. Therefore, memory or priming is a critical aspect governed by epigenetic modifications, imparting an enhanced capacity to combat future stresses (Varotto et al., 2020). In this regard, CRISPR tools like epigenetic modifiers possess immense potential for enabling controlled gene expression by writing, erasing, or reading the epigenetic marks involved in stress-response pathways. Epigenetic modifiers are attractive tools for shedding light on how epigenetic changes may contribute to the development of multi-stress-tolerant crop varieties.

Multiple pathway engineering

Although some CRISPR-based studies have shown that stress-tolerant phenotypes can be generated by targeting single genes, several studies have implied that tolerance to drought and salinity stress may require simultaneous targeting of multiple genes, owing to the polygenic nature of tolerance. Thus, in many cases, single-gene targeting may not be a “silver-bullet” approach to the development of abiotic-stress-resilient crop varieties. Multiple biochemical pathways contribute to plant stress tolerance; therefore, editing a specific gene may affect the functions of proteins in downstream pathways. For example, targeting Na^+ sequestration in vacuoles is of paramount importance to the development of plant salinity tolerance. Targeting vacuolar NHX exchanger genes may not be sufficient to achieve salt-stress tolerance. Therefore, the NHX exchangers must be fueled by a concurrent increase in H^+ -ATPase operation. Second, Na^+ back-leak from the vacuole into the cytosol must be prevented to avoid a futile cycle, and this can be achieved by closely regulating slow and fast vacuolar channels. Thus, simultaneous targeting of multiple genes may enable plant tolerance to salinity. Editing multiple genes from the same or inter-linked pathways would avoid the risk of negative impacts on growth or yield by engineering the plant's tolerance to drought and salinity stresses (Figure 6A). As shown in Figure 6, an additive effect of targeted genes from multiple pathways would contribute to enhanced stress tolerance. Furthermore, sequential editing of several genes involved in a biochemical or signaling pathway can overcome the barrier of rate-limiting steps (as summarized in hypothetical pathway 2 in Figure 6A).

Harnessing natural genetic resources to develop drought/salinity tolerance

CRISPR-based *de novo* domestication of wild species could offer a faster means of crop improvement, as demonstrated in wild tomato (Zsögön et al., 2018) and rice (Yu et al., 2021a, 2021b). Furthermore, crop wild relatives are valuable genetic resources for crop improvement. Comparing susceptible and stress-

tolerant cultivars or wild relatives could explain how multi-level adaptations help tolerant species to survive under stressful conditions. Omics-based comparisons between genotypes with different stress responses is a reliable approach for uncovering uniquely upregulated or downregulated genes/proteins (Figure 6B). Recently, several studies have evaluated drought stress-related parameters in different crops and their wild relatives, such as rice (Neelam et al., 2018), wheat (Liu et al., 2015), sorghum (Cowan et al., 2020), cotton (Yu et al., 2021a, 2021b), tomato (Bolger et al., 2014; Rigano et al., 2016; Egea et al., 2018), eggplant (Kouassi et al., 2021; Plazas et al., 2022), sweet potato (Nhanala and Yencho, 2021), chickpea (Harish et al., 2020), and *Vigna* sp. (Iseki et al., 2018). Drought-tolerant wild sorghum showed higher root dhurrin content than cultivated species (Cowan et al., 2020), and gene expression differences in hormone signaling and amino acid metabolism in wild tomato (*Solanum pennellii*) were crucial for reducing water loss (Egea et al., 2018). Recently, differentially expressed genes or proteins (DEGs/DEPs) were investigated in omics-based drought studies of rice, leading to the identification of genes related to ROS detoxification, signal transduction, cell wall modifications, hormone signaling, transcription regulation, and osmotic adjustment (Baldoni, 2022). Several studies have indicated that the engineering of antioxidative systems may be crucial for drought acclimatization (Laxa et al., 2019). For salt stress in rice, transcriptome and proteome studies have revealed DEGs/DEPs that play a role in ROS detoxification, maintenance of efficient photosynthesis, cell wall integrity, and transport of salt ions across membranes within or outside the cell (Cotsaftis et al., 2011; Li et al., 2018; Farooq et al., 2021). Specific DEGs/DEPs found in rice are depicted in Figure 6B and further elaborated in Supplemental Figure 1. Thus, omics-based identification of stress-responsive genes could provide ideal targets for GE to characterize and further modulate related pathways.

Based on their salt tolerance levels, plants can be categorized as glycophytes (salt sensitive, tolerant of no more than 200 mM NaCl) and halophytes (tolerant of >200 mM NaCl); most cultivated species are glycophytes. Currently, halophyte species are being explored for biotechnology applications that offer several benefits apart from salt tolerance, including pollutant removal, reclamation of degraded lands, and use as high-nutrition fodder or medicinal supplements (Munir et al., 2022). Although glycophytes and halophytes share salt-tolerance mechanisms, the latter have evolved unique features to mitigate higher salt concentrations. For example, halophytes maintain higher osmolyte levels (sugar alcohols, proline) and provide osmoprotection (Gong et al., 2005). Glycophytes are more equipped for higher accumulation of Na^+ in shoots rather than roots while maintaining an optimal K^+/Na^+ ratio (Orsini et al., 2010). The multiple mechanisms of salt tolerance found in *Lycium humile* (tolerant of up to 750 mM NaCl) from the Solanaceae family are attributed to ABA accumulation, higher proline content and antioxidant capacity, and replacement of spongy parenchyma cells with larger parenchyma (water-storage) cells that enable Na^+ storage (Palchetti et al., 2021). In addition, omics approaches have recently been used to investigate the basis of salt tolerance in halophytes (Jiao et al., 2018; Guo et al., 2021). Different omics studies have identified new members or entirely novel gene families implicated in metabolic pathways that help halophytes to cope with a saline

environment; they present ideal targets for GE applications. Although comparative omics approaches provide information about DEGs/DEPs in response to abiotic stresses, further screening of the obtained DEGs/DEPs for GE is a significant challenge. Therefore, an improved understanding of these genes and pathways is critical for informed GE-based crop improvement and can help design and interpret studies of DEGs/DEPs that provide a rich resource for stress-resilient crop development.

Recently, natural allelic variants have been identified in various cultivars through QTL and GWAS approaches. Beneficial allelic variations found in wild relatives can be introduced into elite cultivars using introgressive hybridization. However, this process can be time and labor intensive (Shelake et al., 2019a). Also, there is always a risk of losing some beneficial traits during the breeding process. In this regard, GE tools provide a better means of incorporating beneficial alleles with greater accuracy than introgression breeding, as demonstrated in the case of HAK20 (Wang et al., 2020a) and SOS1 (Wang et al., 2021c) in tomato species.

Engineering detoxification strategies

Stress induces the production of ROS and reactive nitrogen species in different organelles and cell types; these species also function as signaling molecules to activate gene expression and related pathways. Upregulation of antioxidant systems is critical for redox homeostasis during drought stress. For instance, detoxifying enzymes such as ascorbate peroxidase and catalase help to reduce ROS toxicity (Laxa et al., 2019). However, at the moment, differences in the antioxidant systems of sensitive and tolerant genotypes during drought stress are not fully understood, thus limiting GE applications. ROS and related antioxidant systems also play an essential role in plant growth and development, and the spatiotemporal expression patterns of chosen targets for GE must therefore be considered.

Different salt detoxification strategies include salt exclusion at the root-soil interface, maintenance of osmotic balance within cells, sequestration of excess ions in the vacuole, and secretion into specialized salt-gland-like structures (Figure 6C) (Litalien and Zeeb, 2020). Among these, engineering salt-gland-like structures could be an attractive strategy (Figure 6D). Understanding the evolution and function of salt glands may ultimately help to design salt-tolerant crops. Salt glands are unicellular, bicellular, or multicellular structures reported in over 50 halophytes across 14 families (Dassanayake and Larkin, 2017). They originate from the epidermis and structurally resemble plant trichomes. Salt glands in halophytes can be classified into two types on the basis of functional aspects. The first type directly secretes salt ions onto the leaf surface, and the second type stores salt ions in an evolved epidermal bladder cell (EBC) by collecting them into the vacuoles (Figure 6D). The key transporters involved in the EBC have recently been characterized in the halophyte *Chenopodium quinoa* (Bohm et al., 2018). Engineering trichomes into functional salt-gland cells in glycophytes has recently been proposed and discussed in the literature, along with potential candidates for sequestering excess ions in the EBC (Shabala et al., 2014; Dassanayake and Larkin, 2017; Zhao et al., 2020). EBC engineering alone has many limitations and must therefore be

combined with other detoxification strategies to avoid adverse effects on growth and development.

Several recent studies have also explored the potential use of the plant and soil microbiome for enhanced plant tolerance to abiotic stresses, including drought and salinity (Leontidou et al., 2020; An et al., 2022; Koshila Ravi et al., 2022). In the future, combining GE-based approaches with microbial use may constitute an eco-friendly and sustainable approach for higher agricultural crop productivity.

Fine-tuning CRISPR-based tools

Some aspects of CRISPR technology warrant further fine-tuning and upgrades for the application, efficacy, and precision of CRISPR tools in plants; these include selection and in-depth understanding of suitable targets, choice and availability of Cas enzymes, functional sgRNAs without off-target effects in the genome, delivery method of CRISPR reagents, and genotyping of GE lines. Optimization of newer CRISPR tools for different plant species is another challenging endeavor. At first, newer CRISPR-based tools are generally invented for bacterial and mammalian GE, then later adopted for plant use (Shelake et al., 2022). Therefore, further optimizations are required to enable the use of newly developed CRISPR technologies in separate plant cultivars or species.

The editing outcome is mainly dependent on endogenous DNA repair machinery and target locus accessibility in the host genomic architecture. For instance, prime editing is a promising technique for rewriting short DNA lengths, but it is still challenging to use in plants because of lower efficiency (Jiang et al., 2020). Therefore, additional efforts are needed to further improve the PE tool for plant use. Also, current base editing tools cannot install all types of base substitutions at targeted genomic regions. Tools for precisely targeted insertion are another aspect of CRISPR application that requires further improvements, enabling CRE insertions to either activate or suppress the expression of downstream genes (Lu et al., 2020).

Target selection and structural aspects of the sgRNA sequence are also crucial factors in successful GE studies. Chromatin context, DNA repair mechanisms recruited at the targeted sites, expression levels of CRISPR machinery, or a combination of these factors determine the GE outcomes (Weiss et al., 2022). The sgRNA can be designed based on prior knowledge of the genome sequence to avoid off-targets or failure due to several potential factors. In addition, off-targeting can be avoided by backcrossing and segregation (Kadam et al., 2018). In summary, bridging the knowledge gap in drought/salinity-related signaling pathways will help to design GE crops with multiple-stress tolerance and no trade-offs among beneficial traits.

CONCLUDING REMARKS

We have provided an outlook on plant responses to drought and salinity stresses and described the potential use of CRISPR tools along with GAB approaches for the development of stress-resilient crop varieties. Integrated understanding of omics approaches, such as genomics, transcriptomics, proteomics, phenomics, and metabolomics, provides a unique opportunity to

map the flow of genetic information across central dogma processes during stress biology. In this regard, CRISPR technology is a boon for elucidating gene functions and fine-tuning plant response pathways for tolerance to drought, salinity, and other stresses in agriculture. The expanding CRISPR toolbox enables the targeting of any process that regulates plant traits by precise and efficient genome engineering. In the near future, CRISPR tools will be a valuable resource helping plant biologists to engineer stress-resistant crop varieties for changing climatic conditions.

SUPPLEMENTAL INFORMATION

Supplemental information is available at *Plant Communications Online*.

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AUTHOR CONTRIBUTIONS

R.M.S. and J.-Y.K. conceived the idea. R.M.S. researched and designed the manuscript structure. R.M.S. wrote the initial draft. U.S.K., R.K., D.P., and A.K.S. contributed to writing and editing. All authors made direct intellectual contributions to the editing before final submission and approved the manuscript.

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Plant Communications, Volume 3

Supplemental information

**Engineering drought and salinity tolerance traits in crops through
CRISPR-mediated genome editing: Targets, tools, challenges, and
perspectives**

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Supplementary information 1

Introgression of drought and salinity tolerance traits in crops through CRISPR-based precision genome engineering

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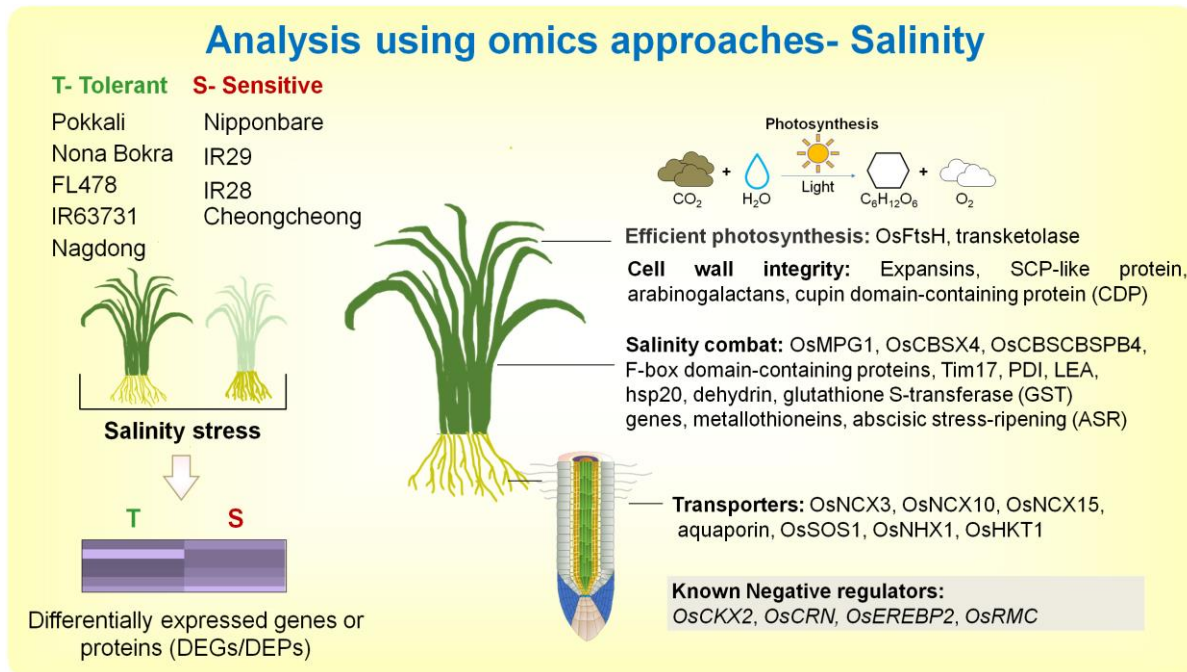


Figure S1. Deciphering uniquely expressed genes in salt-stress tolerance using omics approaches is summarized for rice studies. Comparative omics studies between salinity tolerant and sensitive varieties have been performed to find the differentially expressed genes or proteins (DEGs or DEPs) [Cotsaftis et al., 2011- Pokkali (T), Nona Bokra (T), Nipponbare (S); Li et al., 2018- Pokkali (T), IR-29 (S); Farooq et al., 2021- Pokkali (T), Nagdong (T), IR-28 (S), Cheongcheong (S)]. Comparative transcriptomics and translato-me data predicted several unique DEGs/DEPs that could be combined precisely to improve the salinity tolerance in salt-sensitive commercial rice cultivars. In the above-ground part of the plant, photosynthesis is mainly affected by salinity stress. FtsH-like chloroplast proteins (Os02g43350 and Os05g38400) were highly accumulated during the salinity stress, and these proteins are associated with the photosynthetic electron transport chain (Li et al., 2018). Similarly, transketolase (Os04g19740) was highly expressed. Transketolase enzyme transfers a residue with two carbon atoms from fructose-6-phosphate to glyceraldehydes-3-phosphate, resulting in ribulose-1,5-bisphosphate (RuBP) formation. Both FtsH-like chloroplast proteins and transketolase could be modified and expressed differentially to mitigate the effects of salinity stress on photosynthesis. Cell wall integrity is essential in the cell defense from high salt accumulation. Many genes were upregulated in the salt-tolerant Pokkali land race, which includes arabinogalactan proteins, expansins, SCP-like proteins, and cupin domain-containing proteins (CDPs). Ion transporters are involved in the intrusion and extrusion of Na^+ during elevated salinity stress. Many transporters have been explicitly accumulated in the Pokkali. For instance, transporters like *OsNCX3*, *OsNCX10*, *OsNCX15*, *Aquaporins*, *OsSOS1*, *NHX1*, and *OsHKT1* are the best candidates for reorganizing the Na^+ transport (Singh et al., 2015; Li et al., 2018). Specific transporters could be targeted to increase the extrusion or block the Na^+ intrusion. Many known downstream targets help in maintaining cellular homeostasis during salinity stress. The transcripts of the genes like *OsMPG1* (Kumar et al., 2012), *OsCBSX4* (Singh et al., 2012), and *OsCBSCBSPB4* (Kumar et al., 2018), were highly accumulated during salinity stress in Pokkali as compared to IR64 and their overexpression in tobacco has shown tolerance to the salinity stress. Further detailed study of the mode of action of these genes could help in exploring novel genes or pathways to alleviate the salinity stress. Similarly, Pokkali-specific upregulation of F-box domain-containing proteins, Tim17, PDI, LEA, hsp20, dehydrin, Glutathione S-transferase (GST) genes, metallothioneins, and Abscisic stress-ripening (ASR)

was found in comparison to salt-sensitive IR29 variety (Li et al., 2018). These genes could be a strong candidate for tackling salinity stress. DNA methylation modulates the pre-transcriptional changes during stress. Analysis of methylation profile in salt-tolerant and susceptible rice varieties showed specific changes in Pokkali (Farooq et al., 2021). The DNA demethylases and methyltransferases were highly induced in the Pokkali compared to IR29. Further, the plasticity of DNA methylation could be tamed to generate the salt-tolerance traits in the salt-sensitive varieties. Most targets mentioned above are positive regulators and need overexpression or activation by CRISPRa-type systems. On the other hand, CRISPR/Cas9-mediated knockouts of negative regulators like *OsCKX2* (Joshi et al., 2018), *OsCRN* (Kojonna et al., 2022), *OsEREBP2* and *OsRMC* (Serra et al., 2013) could assist in producing salt-tolerant rice genotypes.

Supplementary Table S1. Abbreviations of transcription factors (TFs), genes, and protein families.

| Abbreviation | Description |
|---------------------|-------------------------------------------------------------------|
| ABRE | ABA-responsive element family |
| <i>ACQOS</i> | <i>Acquired osmotolerance</i> |
| <i>AITR</i> | <i>ABA-induced transcription repressors</i> |
| <i>AIW1, AIW2</i> | <i>ABA-induced WD40-repeat 1 and 2</i> |
| AP2/ERF | APETALA2/ethylene-responsive TF factor family |
| AREB/ABF | ABRE-binding protein/ABRE binding factor family |
| AREB1 | ABA-responsive element-binding protein 1 family |
| <i>ARF4</i> | Auxin response factor 4 |
| <i>ARGOS8</i> | <i>Auxin-regulated gene involved in organ size 8</i> |
| AVP1 | <i>Arabidopsis</i> vacuolar H ⁺ -pyrophosphatase |
| <i>BBS1</i> | Bilateral blade senescence 1 |
| <i>BG3</i> | <i>Big grain3</i> |
| bHLH | Basic helix-loop-helix family |
| bZIP | Basic leucine zipper protein family |
| <i>C/VIF1</i> | <i>Cell wall/vacuolar inhibitor of fructosidase 1</i> |
| CDF | Cycling DOF Factor family |
| CDPK | Calcium-dependent protein kinase family |
| CIPK | Calcineurin B-like-interacting protein kinase family |
| <i>CLCg</i> | <i>Voltage-gated chloride channel</i> family |
| <i>DOF15</i> | DNA-binding with one finger 15 |
| DPA4 | <i>Development-Related PcG Target in the APEX4</i> |
| DREB | Dehydration responsive element binding protein family |
| <i>DREB</i> | <i>Dehydrin, 1,8-dihydroxy naphthalene</i> family |
| <i>DST</i> | <i>drought and salt tolerance</i> family |
| <i>ELF4</i> | <i>Early flowering 4</i> |
| ENA | Na ²⁺ -extrusion family |
| <i>ERA1</i> | Enhanced Response to ABA1 |
| <i>ERF83</i> | <i>Ethylene-responsive factor83</i> |
| <i>FLN2</i> | Fructokinase-like2 |
| <i>GI</i> | <i>GIGANTEA</i> family |
| <i>GID1a</i> | <i>Gibberellin-insensitive dwarf1a</i> |
| <i>GNC</i> | <i>GATA, nitrate-inducible, carbon-metabolism-involved</i> family |
| <i>GST</i> | <i>Glutathione S-transferase</i> family |
| <i>GTγ-2</i> | Trihelix transcription factor γ-2 |

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|----------------------|-----------------------------------------------------------------------------------------------------------------|
| <i>HAG1</i> | <i>Histone acetyltransferase</i> |
| <i>HAK20</i> | <i>High-affinity K⁺ transporter</i> |
| HD-Zip | Homeodomain-leucine zipper family |
| HKT | High-affinity K ⁺ transporter family |
| <i>HKT1</i> | <i>High-affinity K⁺ transporter1</i> |
| <i>HVP10</i> | <i>Vacuolar H⁺-pumping pyrophosphatase 10</i> |
| <i>HyPRP1</i> | <i>Hybrid proline-rich protein 1</i> |
| <i>ITPK1</i> | <i>Inositol trisphosphate 5/6 kinase 1</i> |
| KCO | TPK/K ⁺ channel outward channels family |
| KT/HAK/KUP | K ⁺ transporter (KT)/high-affinity K ⁺ (HAK)/ K ⁺ uptake permease (KUP) family |
| <i>LBD40</i> | <i>Lateral organ boundaries domain 40</i> |
| LRR-XII | Leucine-rich repeat receptor-like kinase subfamily XII |
| MAPK | Mitogen-activated-protein-kinase family |
| <i>MOCA1</i> | <i>Monocation-induced (Ca²⁺) increases 1</i> |
| MYB | Myeloblastosis family |
| NAC | NAM, ATAF1-2, and CUC2 family |
| <i>NCA1a/OsNCA1b</i> | <i>No catalase activity 1a and b</i> |
| NHX | Na ⁺ /H ⁺ or K ⁺ /H ⁺ exchanger family |
| <i>NPR1</i> | <i>Non-expressor of pathogenesis-related gene 1</i> |
| <i>OSCA1</i> | <i>reduced hyperosmolality-induced Ca²⁺ increase 1</i> |
| <i>OST2</i> | <i>OPEN STOMATA 2</i> |
| <i>PdNF-YB21</i> | <i>Root-specific nuclear factor Y</i> |
| <i>PIL14</i> | <i>Phytochrome-interacting factor-like14</i> |
| <i>PQT3</i> | <i>Paraquat tolerance 3</i> |
| <i>RAV2</i> | <i>Related to ABI3/VP1</i> |
| <i>RBOHD</i> | <i>Respiratory burst oxidase homolog D</i> |
| <i>RR22</i> | <i>B-type response regulator 22</i> |
| <i>RR9, RR10</i> | <i>Type-A response regulator 1 and 2</i> |
| <i>SAPK1, SAPK2</i> | <i>stress/ABA-activated protein kinase 1 and 2</i> |
| <i>SAUR41</i> | <i>Small auxin up RNA 41</i> |
| SnRK | SNF1-related protein kinase family |
| SOD7 | <i>Suppressor of da1-1</i> |
| SOS | <i>Salt overly sensitive antiporter family</i> |
| <i>SPL10</i> | <i>Squamosa promoter-binding protein-like 10</i> |
| <i>SRL1, 2</i> | <i>Semi-rolled leaf 1 and 2</i> |
| <i>UGT</i> | <i>UDP-glycosyltransferase</i> |
| VGICs | Non-voltage-gated (tandem pore K ⁺) channels |
| WDR | (WD40-repeat) family |

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| WHY | WHIRLY family |
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Supplementary Table S2. The Cas orthologs adopted for CRISPR-based tool development and used to plot the Figure 3.

| Ortholog | PAM (5'-3') | Source | Feature | Reference |
|-------------------------|-----------------|--------------------------------------------|------------------------|--------------------------------------------|
| SpCas9 | NGG | <i>Streptococcus pyogenes</i> | Most used Cas ortholog | (Jinek et al., 2012) |
| St1Cas9 | NNAGAAW | <i>Streptococcus thermophilus</i> | Cas ortholog | (Cong et al., 2013) |
| St3Cas9 | NGGNG | <i>Streptococcus thermophilus</i> | Cas ortholog | (Cong et al., 2013) |
| NmeCas9 | NNNNGMTT | <i>Neisseria meningitidis</i> | Cas ortholog | (Hou et al., 2013) |
| TdCas9 | NAAAAN | <i>Treponema denticola</i> | Cas ortholog | (Esvelt et al., 2013) |
| FnCas9 | NGG | <i>Francisella novivida</i> | Cas ortholog | (Fonfara et al., 2014; Price et al., 2015) |
| SaCas9 | NNNRRT | <i>Staphylococcus aureus</i> | Cas ortholog | (Ran et al., 2015) |
| BlatCas9 | NNNNCND | <i>Brevibacillus laterosporus</i> | Cas ortholog | (Karvelis et al., 2015) |
| FnCpf1 | TTV/TTTV/KY TV | <i>Francisella tularensis</i> | Cas ortholog | (Zetsche et al., 2015) |
| LbCpf1 | TTTV | <i>Lachnospiraceae bacterium ND2006</i> | Cas ortholog | (Zetsche et al., 2015) |
| AsCpf1 | TTTV | <i>Acidaminococcus</i> sp. BV3L6 | Cas ortholog | (Zetsche et al., 2015) |
| BpCpf1 | - | <i>Butyrivibrio proteoclasticus</i> | Cas ortholog | (Zetsche et al., 2015) |
| SsCpf1 | - | <i>Smithella</i> sp. SC_K08D17 | Cas ortholog | (Zetsche et al., 2015) |
| Cas13a | - | <i>Leptotrichia shahii</i> | RNA targeting | (Abudayyeh et al., 2016) |
| CjCas9 | NNNVRYAC | <i>Campylobacter jejuni</i> | Smallest Cas ortholog | (Kim et al., 2017) |
| ThermoCas9 | NNNNCNR | <i>Geobacillus thermodenitrificans T12</i> | Cas ortholog | (Mougiakos et al., 2017) |
| Cas13b | - | <i>Bergeyella zoohelcum</i> | RNA targeting | (Smargon et al., 2017) |
| ScCas9 | NNG | <i>Streptococcus canis</i> | Cas ortholog | (Chatterjee et al., 2018) |
| MbCpf1 | TTV/TTTV | <i>Moraxella bovoculi 237</i> | Cas ortholog | (Tóth et al., 2018) |
| Cas13d | - | <i>Eubacterium siraeum</i> | RNA targeting | (Koner mann et al., 2018) |
| CasX | TTCN | Uncultured microbial communities | Cas ortholog | (Liu et al., 2019) |
| Cas12f (Cas14) homologs | 5' T- or C-rich | Different species | Cas ortholog | (Karvelis et al., 2020) |
| SmacCas9 | NAAN | <i>Streptococcus macacae</i> | Cas ortholog | (Chatterjee et al., 2020a) |
| ErCpf1 (MAD7) | YTTN | <i>Eubacterium rectale</i> | Royalty-free ortholog | (I. Inscripta, 2020) |
| CeCpf1 | TTTV | <i>Coprococcus eutactus</i> | Cas ortholog | (Chen et al., 2020) |
| BfCpf1 | TTTV | <i>Butyrivibrio fibrisolvens</i> | Cas ortholog | (Chen et al., 2020) |

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|---------------|--------|-----------------------------------|--------------|------------------------|
| CasΦ (Cas12j) | TBN | Bacteriophage | Cas ortholog | (Pausch et al., 2020) |
| ShyCas9 | NNARMM | <i>Staphylococcus hyicus</i> | Cas ortholog | (Schmidt et al., 2021) |
| SluCas9 | NNGG | <i>Staphylococcus lugdunensis</i> | Cas ortholog | (Schmidt et al., 2021) |
| SmiCas9 | NNGG | <i>Staphylococcus microti</i> | Cas ortholog | (Schmidt et al., 2021) |
| SpaCas9 | NNGG | <i>Staphylococcus pasteurii</i> | Cas ortholog | (Schmidt et al., 2021) |

N:any nucleotide, R:A/G, M:A/C, W:A/T, V:G/C/A, Y:C/T, H:A/C/T, B:G/T/C.

Supplementary Table S3. The Cas variants adopted for CRISPR-based tool development and used to plot the Figure 3.

| Variants | PAM (5'-3') | Mutation | Feature | Reference |
|----------------|--------------|--------------------------------------------------|----------------------|----------------------------|
| SpCas9(D1135E) | NAG, NGA | D1135E | Relaxed PAM | (Kleinstiver et al., 2015) |
| SpCas9(VQR) | NGA | D1135V/R1335Q/T1337R | Relaxed PAM | (Kleinstiver et al., 2015) |
| SpCas9(EQR) | NGAG | D1135E/R1335Q/T1337R | Relaxed PAM | (Kleinstiver et al., 2015) |
| SpCas9(VRER) | NGCG | D1135V/G1218R/R1335E/T1337R | Relaxed PAM | (Kleinstiver et al., 2015) |
| SaCas9 KHH | NNNRRT | E782K/N968K/R1015H | Relaxed PAM | (Kleinstiver et al., 2015) |
| SpCas9(K855A) | NGG | K855A | Enhanced specificity | (Slaymaker et al., 2016) |
| eSpCas9(1.0) | NGG | K810A/K1003A/R1060A | Enhanced specificity | (Slaymaker et al., 2016) |
| eSpCas9(1.1) | NGG | K848A/K1003A/R1060A | Enhanced specificity | (Slaymaker et al., 2016) |
| SpCas9-HF1 | NGG | N497A/R661A/Q695A/Q926A | Enhanced specificity | (Kleinstiver et al., 2016) |
| SpCas9 QQR1 | NAAG | G1218R/N1286Q/I1331F/D1332K/R1333Q/R1335Q/T1337R | Relaxed PAM | (Anders et al., 2016) |
| HeFSpCas9 | NGG | N497A/R661A/Q695A/K848A/Q926A/K1003A/R1060A | Enhanced specificity | (Kulcsár et al., 2017) |
| HypaCas9 | NGG | N692A/M694A/Q695A/H698A | Enhanced specificity | (Chen et al., 2017) |
| AsCpf1(RR) | TYCV, CCCC | S542R/K607R | - | (Gao et al., 2017) |
| AsCpf1(RVR) | TATV | S542R/K548V/N552R | - | (Gao et al., 2017) |
| LbCpf1(RR) | TYCV, CCCC | G532R/K595R | Relaxed PAM | (Gao et al., 2017) |
| LbCpf1(RVR) | TATV | G532R/K538V/Y542R | Relaxed PAM | (Gao et al., 2017) |
| evoCas9 | NGG | M495V/Y515N/K526E/R661Q | Enhanced specificity | (Casini et al., 2018) |
| HiFi Cas9 | NGG | R691A | Enhanced specificity | (Vakulskas et al., 2018) |
| Sniper Cas9 | NGG | F539S/M763I/K890N | Enhanced specificity | (Lee et al., 2018) |
| eHF1-Cas9 | NGG | N497A/R661A/Q695A/K848A/Q926A/K1003A/R1060A | Enhanced specificity | (Liang et al., 2018) |
| eHypaCas9 | NGG | N692A/M694A/Q695A/H698A/K848A/K1003A/R1060A | Enhanced specificity | (Liang et al., 2018) |
| xCas9 3.7 | NG, GAA, GAT | A262T/R324L/S409I/E480K/E543D/M694I/E1219V | Relaxed PAM | (Hu et al., 2018) |
| SpCas9-NG | NG | R1335V/L1111R/D1135V/G1218R/E1219F/A1322R/T1337R | Relaxed PAM | (Nishimasu et al., 2018) |
| FnCpf1(RVR) | TYCV, TCTV | N607R/K671R | - | (Tóth et al., 2018) |
| FnCpf1(RR) | TWTV | N607R/K613V/N617R | - | (Tóth et al., 2018) |
| MbCpf1(RR) | TYCV, TCTV | N576R/K637R | Relaxed PAM | (Tóth et al., 2018) |
| MbCpf1(RVR) | TWTV | N576R/K582V/N586R | Relaxed PAM | (Tóth et al., 2018) |

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|-------------------------|------------------------|------------------------------------------------------------------------------|-------------------------|------------------------------|
| enAsCpf1 | VTTV, TTTT, TTCN, TATV | E174R/S542R/K548R | Temperature insensitive | (Kleinstiver et al., 2019) |
| iSpmacCas9 | NAA | R221K/N394K | Relaxed PAM | (Chatterjee et al., 2020a) |
| SpCas9-NRRH | NRRH | R1114G/D1135N/V1139A/D1180G/E1219V/Q1221H/A1320V/R1333K | Relaxed PAM | (Miller et al., 2020) |
| SpCas9-NRTH | NRTH | R1114G/D1135N/D1180G/G1218S/E1219V/Q1221H/P1249S/E1253K/P1321S/D1332G/R1335L | Relaxed PAM | (Miller et al., 2020) |
| SpCas9-NRCH | NRCH | R1114G/D1135N/E1219V/D1332N/R1335Q/T1337N/S1338T/H1349R | Relaxed PAM | (Miller et al., 2020) |
| ScCas9-Sc ⁺⁺ | NNG | T1227K | Relaxed PAM | (Chatterjee et al., 2020b) |
| HiFi-Sc ⁺⁺ | NNG | R701A/T1227K | Relaxed PAM | (Chatterjee et al., 2020b) |
| SpG | NGN | D1135L/S1136W/G1218K/E1219Q/R1335Q/T1337R | Relaxed PAM | (Walton et al., 2020) |
| SpY | NRN, NYN | A61R/L1111R/D1135L/S1136W/G1218K/E1219Q/N1317R/A1322R/R1333P, R1335Q/T1337R | Relaxed PAM | (Walton et al., 2020) |
| enLbCpf1 | TTTV | D156R/G532R/K538R | Temperature insensitive | (Schindele and Puchta, 2020) |
| ttLbCpf1 | TTTV | D156R | Temperature tolerance | (Schindele and Puchta, 2020) |
| enCjCas9 | NNNVRVYAC | L58Y/D900K | Enhanced specificity | (Nakagawa et al., 2021) |

N:any nucleotide, R:A/G, M:A/C, W:A/T, V:G/C/A, Y:C/T, H:A/C/T, B:G/T/C.

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