

Genome editing for enhancing production and productivity of maize in India

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Abstract Maize is the third most important crop in India being cultivated in 10.2 million hectares of land. In India, maize is mainly a rainfed crop with nearly 80% area under rainfed ecology, where the productivity is very low and is affected mainly by drought stress due to long dry spells. Therefore increasing maize yield in said ecology is a major challenge. Furthermore, it is estimated that an additional ~10 million tonnes of maize kernel would be required by 2025-26 to achieve the E20 target (20% bioethanol blending of petrol) envisaged by the government. Taken together, it is imperative to develop high-yielding as well as climate-resilient Indian maize genotypes for yield enhancement and sustainability. In this endeavor, genome editing tools should be employed to develop such genotypes rapidly. Here we enlisted four target genes (*KRN2*, *RAV1*, *ABH2*, and *DST*) which are major regulators of key biological functions such as kernel row number per plant, leaf inclination angle imparting the erect leaf phenotype, drought, and salt tolerance. It is expected that knocking out these genes would improve overall yield by increasing kernel numbers per ear, plant population per unit area, as well as confer tolerance to drought and salinity. Besides, an erect leaf phenotype that is suitable for high-density planting will also contribute to drought tolerance by reducing transpiration water loss, improving photosynthesis rate, and resource use efficiency. With these interventions, it is expected that maize grain yield would be improved and

thus help in addressing the twin challenges of feed and fuel security.

Keywords: Kernel number · Drought and salt tolerance · Leaf angle · Erect leaf · High-density planting · Maize grain yield

Background

Maize is a source of feed, food, industrial raw material, and livelihood for millions of farmers in India. Its demand is likely to increase due to the ever-increasing population and to attain the E20 and E30 blending target of the Government of India (GOI) by 2025-26 and 2030, respectively, where NITI Aayog has identified maize as the most important feedstock for bioethanol production. The average maize productivity in India (~3.2 t/ha) is still much below the global average (~5.7 t/ha) (FAOSTAT, 2021; FICCI Vision, 2022). This is because approximately 80% of maize (~8 mha) is grown under rainfed ecology (*kharif* season) in our country where yield is even below (~2.7 t/ha) the national average (DACNET, 2020). The unpredictable and long dry spells leading to drought stress cause huge yield penalties often in the range of 35-66% (Kumar *et al.*, 2016; Sah *et al.*, 2020). Therefore, drought stress drastically hampered the maize productivity in the rainfed ecology. Meeting the increasing demand for maize in our country would require increasing the area under cultivation of maize as well as enhancing its productivity under sub-optimal environmental conditions. In view of the above, it is essential to develop high-yielding as well as climate-resilient maize genotypes to increase yield and promote sustainability. Kernel number per plant is the most

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important trait contributing to maize grain yield. Additionally, it has been proven that increasing planting density by reducing leaf inclination angle is an effective strategy to increase maize yield. Considering the above facts, it would be ideal to develop tropical maize genotypes having higher kernels, erect leaf architecture suitable for high-density planting, and tolerance to drought and salinity. Such genotypes could be developed by loss-of-function of the major regulator(s) of these yield-contributing traits using CRISPR/Cas9-mediated targeted mutagenesis. Four such well-characterized regulators/genes are *ZmKRN2* governing kernel rows per plant, *ZmRAVL1* regulating leaf inclination angle, *ZmABH2* regulating stomatal closure under water deficit conditions, and *ZmDST* regulating stomatal closure under drought as well as salt stress.

For imparting higher kernel numbers per plant *ZmKRN2* gene encoding WD40 protein should be knocked out. The kernel row number 2 (*ZmKRN2*) gene is a major negative regulator that governs kernel rows per plant. Recently, it has been shown that knocking out the *KRN2* gene – which underlies a major QTL for kernel row number – increases maize yields by 10% due to increasing kernel number/plant under field conditions with no apparent trade-off in other agronomic traits (Chen *et al.*, 2022).

For developing plant ideotypes with upright/erect leaf orientation *ZmRAVL1* gene encoding the B3-domain transcription factor should be knocked out. A loss-of-function mutant of *ZmRAVL1* has been shown to reduce leaf inclination angle by nearly 25-45% (upright leaves) without any detrimental effect which reduces plant spacing and thereby accommodating more plants per unit area (Tian *et al.*, 2019).

To confer drought and salt tolerance, two genes namely *ZmABH2* encoding an abscisic acid 82 -hydroxylase (ABAox) and maize ortholog of rice drought and salt tolerance (*DST*) gene-which are the major negative regulators of drought and salt tolerance and have a well-established proof-of-concept in maize and rice, respectively (Liu *et al.*, 2020; Santosh Kumar *et al.*, 2020) -could be knocked out using CRISPR/Cas9 approach.

Improving kernel row number

Enhancing the production and productivity of Indian tropical maize is needed to meet its continuously

increasing demand for poultry feed, biofuel production, and other industrial uses. To date, the conventional breeding approach has been used for maize yield improvement, and many single cross-hybrids have been developed and deployed for the same. Although conventional breeding has contributed tremendously in terms of yield enhancement, still, there is still scope to enhance yield as the average maize yield (3.2 tons/ha) is much less than the global average (~5.7 t/ha) (FAOSTAT, 2021).

The kernel number per row (KNR) of maize is a key trait that contributes greatly to grain yield per ear (Cai *et al.*, 2014). The formation of kernel rows is an integral part of the development of the female inflorescence in maize. Over the last few decades, many genes that determine inflorescence development and architecture have been identified and characterized. In a recent breakthrough study, it has been shown that knocking out the *KRN2* gene increases maize yields by 10% via regulating inflorescence meristem size resulting in higher kernel number/plant under field trials with no apparent pleiotropic effects (Chen *et al.*, 2022). Similarly, the rice ortholog, *OsKRN2* negatively regulates grain number via the control of secondary panicle branches. Disrupting this gene in rice results in a nearly 8% higher yield under field conditions without a negative impact on other agronomic traits (Chen *et al.*, 2022). Furthermore, transgenic overexpression of *KRN2* significantly decreases grain yield in maize and rice. As *ZmKRN2* is a major negative regulator of kernel row number, hence, it is likely to be a good molecular target for increasing kernel row number and hence grain yield in tropical Indian maize via generating loss-of-function mutants using the genome-editing approach (Figure 1).

Suitable plant architecture for high-density planting

Apart from increasing the kernel number per plant, growing more plants per unit area (high planting density) can be another effective strategy to achieve higher productivity. Over the decades, the deployment of high plant density has led to a significant gain in temperate maize yield (Cao *et al.*, 2022). However, in Indian tropical maize, the potential of this trait is not fully harnessed due to low germplasm diversity (Sandhu and Dhillon, 2021).

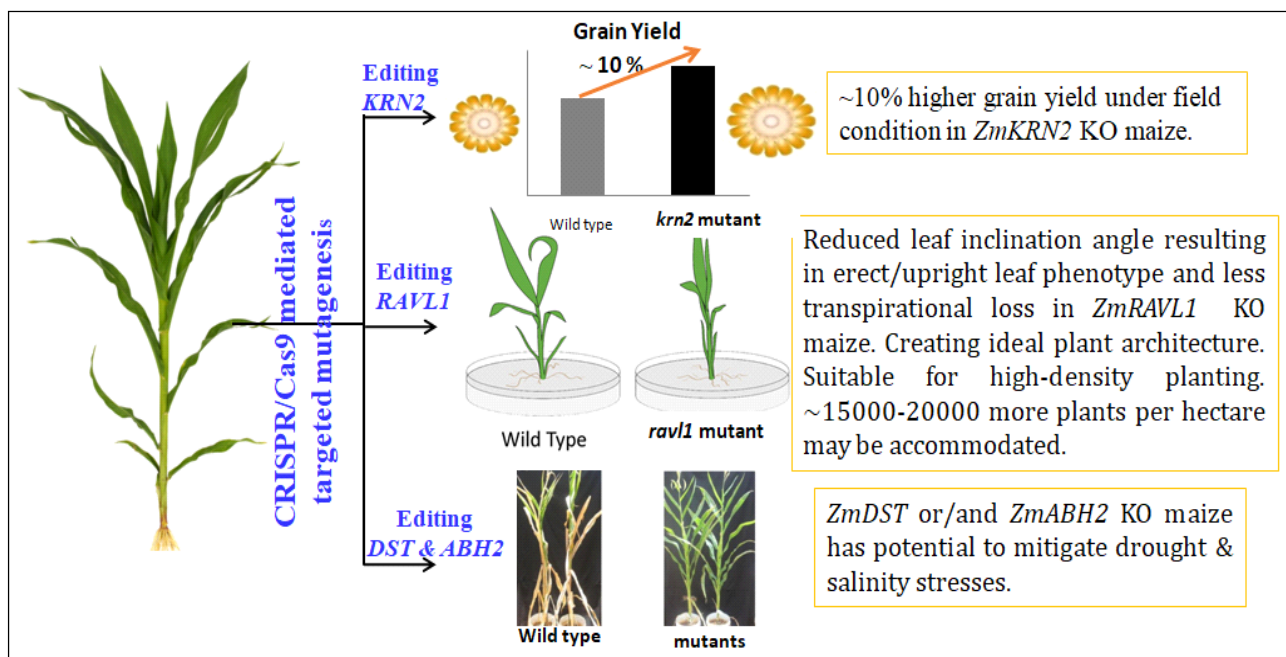


Figure 1. Schematic representation of potential outcomes of precise editing of four major regulatory genes i.e. *ZmKRN2*, *ZmRAVL1*, *ZmABH2*, and *ZmDST* in tropical Indian maize. These genes regulate yield-contributing traits such as kernel row number per ear, leaf inclination angle, drought tolerance, and drought as well as salt tolerance, respectively. Adapted from Brant *et al.* 2021; Chen *et al.* 2022.

Upright plant architecture (smaller leaf angle) facilitates dense planting. Plant architecture with more upright leaves decreases mutual shading and sustains light capture for photosynthesis despite increased plant density, thus improving the accumulation of leaf nitrogen for grain filling and increasing grain yield. Therefore, leaf angle is an important agronomic trait determining maize planting density and light penetration into the canopy and contributes to grain yield. The establishment of the leaf angle in maize is largely determined by the ligular region, which encompasses the ligule and auricle and serves as a hinge at the blade-sheath boundary, allowing the leaf blade to project away from the stem.

In a recent breakthrough study, Tian *et al.* (2019) cloned the causal genes underlying two QTLs, *UPRIGHT PLANT ARCHITECTURE 1 (UPA1)* and *UPA2* - found in some rare accessions of teosinte and was lost during domestication - that confer upright architecture. Authors demonstrated that the causal gene i.e. *ZmRAVL1* (GRMZM2G102059), encoding a B3 domain-containing protein, directly activates *ZmBRD1* expression, leading to increased Brassinosteroids levels and larger leaf angle. Consistently, *ZmRAVL1* positively regulates leaf angle, as disrupting this gene reduces leaf angle while overexpressing this gene increases leaf angle., *ZmRAVL1*-knockout lines exhibited normal ligular development and

only affected auricle expansion thereby reducing leaf angle by nearly 25-45% without any other detrimental effect. Field trials have shown that CRISPR edited *ZmRAVL1* allele enables dense planting (105000 plants per hectare) and increases yield significantly (Tian *et al.*, 2019). Thus, disrupting the expression of this gene has been proven to enhance high-density maize yields under field conditions. In India, most of the tropical maize germplasm has very high leaf angle /drooping type leaf and we are lagging behind the USA and China in harnessing the potential of upright leaf architecture trait for high-density planting. Creating an ideal plant architecture/ideotype having an upright/erect leaf suitable for tolerating high-density planting stress holds immense potential for maize yield enhancement. Since *ZmRAVL1* has been proven as a major positive regulator of leaf angle, hence it is an ideal target for creating optimal maize architecture suitable for dense planting (Figure 1). Additionally, the erect leaf phenotype is relatively drought tolerant due to less transpiration loss which would be again beneficial in rainfed ecology.

Abiotic stresses tolerance

In India over 80% of *kharif* maize area is predominantly rainfed where crops are susceptible to the erratic behavior

of rains. Drought during pre-flowering and grain-filling stages massively affects the plant performance due to imprecise trait function. Thus, *kharif* maize in India is subjected to the vagaries of climate leading to low productivity (2.70 t/ha) against rabi maize, with average productivity of 4.43 t/ha (DACNET, 2020). Drought stress has severely hampered maize production, in such ecologies, affecting the livelihood and economics of millions of people.

Under the crop diversification plan of the GOI for the sustainability of crop production systems, rice cultivation in some parts of Indo-Gangetic Plains (Haryana and Punjab) and upland rice growing areas are to be replaced with maize crops. However, soil in these areas is mostly affected by high salinity due to the prevailing practice of flood irrigation. Therefore, salt-tolerant maize is the need of the hour to make crop diversification successful in such areas. Thus, developing salt-tolerant maize would help in making maize cultivation more profitable than rice cultivation in such areas. Taken together, there is an urgent need to develop maize varieties that have a high degree of tolerance to drought and salt stress. However, to the best of our knowledge, neither drought-tolerant nor salt-tolerant hybrids have been developed so far in the Indian maize improvement program.

Drought tolerance is a polygenic and complex trait and to date, several genes have been shown to play a role in drought tolerance in plants (Shelake *et al.*, 2022). Many QTLs and candidate genes playing a key role in drought and salinity stress adaptation are identified in maize and other crops globally. Most of the previous studies are based on a transgenic/GM approach which limits their widespread acceptance/adoption due to the presence of foreign gene(s). In maize, recently a few studies have been carried out to improve drought tolerance by utilizing CRISPR/Cas technology (Shi *et al.*, 2017). After surveying published literature, we narrowed down two well-characterized major negative regulators namely *ZmABH2* and *DST* imparting drought and drought as well as salinity tolerance in maize and rice, respectively (Cui *et al.*, 2015; Lui *et al.*, 2020).

Under drought stress, Absciscic acid (ABA) mediates the stomatal closure and thereby reduces water loss by transpiration. ABA stimulates root cell elongation and enables plants to uptake water from water-deficit soil (Aslam *et al.*, 2022). One drought-responsive gene, *ABH2*

encoding an abscisic acid 8'-hydroxylase (ABA Ox) that is involved in the first step in the oxidative degradation of ABA has been proven to negatively regulate drought tolerance. ABAox regulates stomatal opening in response to high humidity (Liu *et al.*, 2020). The *ABH2* knock-out maize has 30 % higher ABA content than wild-type maize plants. Disruption of the *ABH2* gene in maize results in quicker stomatal closure in response to dehydration stress and thereby confers significantly higher drought tolerance through modulating ABA content under water deficit (Liu *et al.*, 2020) (Figure 1).

Another well-characterized major negative regulator governing drought and salt tolerance in rice is the *DST* gene, encoding a zinc finger transcription factor. Huang *et al.* (2009) reported that N69D mutation in the *DST* gene results in loss of function (*dst* mutant) which leads to drought and salt tolerant phenotype in rice. The mutant rice lines exhibit higher leaf width, lower stomatal density, and reduced transpiration (Huang *et al.*, 2009). In another study, a single nucleotide insertion mutation in the *DST* gene (*DST^{reg1}*) enhanced the cytokine level through the inactivation of *Gn1a/OsCKX2* (Grain number 1a/Cytokinin Oxidase 2) gene. The enhanced cytokine level in the reproductive meristem leads to increased panicle branching and grain number in rice (Li *et al.*, 2013). More recently, CRISPR/Cas9-mediated knockout of this gene in indica rice has been shown to exhibit moderate drought and high salinity tolerance (Kumar *et al.*, 2020). *OsDST* gene has been shown to negatively regulate stomatal closure by direct modulation of genes related to H₂O₂ homeostasis. The *DST* mutant rice lines have enhanced leaf width and reduced stomatal density (Santosh Kumar *et al.*, 2020). Thus, *ABH2* and *DST* are two well-proven negative regulators of drought, and drought as well as salt tolerance in maize and rice, respectively. Further, the *DST* gene is conserved in major cereals. Considering these facts, generating loss-of-function mutants of these genes is expected to impart drought and salt tolerance in the Indian tropical maize (Figure 1). Salt-tolerant maize genotype would be beneficial for crop diversification.

Conclusion

In summary, *ZmKRN2*, *ZmRAVL1*, *ZmABH2*, and *ZmDST* genes are major negative regulator genes having well-established proof-of-concept and thereby potentially good targets for knocking out their expression in Indian maize

via precise genome editing tools (SDN-1), and the same has not yet been explored in tropical Indian maize. Developing genome-edited lines carrying a loss-of-function mutation in these genes will lead to increased kernel number/ear, plants suitable for high-planting density, drought, drought as well as salt tolerance, respectively. The transgene-free gene-edited lines i.e. knockout mutants developed could be utilized as donors in the breeding programme to develop high-yielding drought and salt-tolerant hybrids for different ecologies. Doing such interventions would be a landmark contribution to the Indian maize improvement program. Besides, globally these traits have not been stacked so far which has the potential to further enhance maize yield significantly under field conditions. Thus, in the long-term, the development of such genotypes would result in the increase of maize productivity and production in India which in turn will improve the income and livelihood of the farmers and other maize-based stakeholders.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contribution statement

KK and BK conceptualized the idea. KK wrote the manuscript. AKJ, PP and Neha contributed to the preparation schematic diagram. HSJ and BK contributed to critically revising the draft. All authors read and approved the manuscript.

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