

PERSPECTIVE

## Allelic value in gene regulation—implications for gene editing

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- **Background** Gene editing has emerged as the most precise trait modification tool in plant breeding. However, an understanding of what to target and in which genetic background to obtain the intended phenotype is still emerging. This perspective presents an analytical overview of traits targeted, particularly in tomato and rice, where extensive data on gene editing are available in the public domain.
- **Scope** The available gene editing data on allelic values for a given molecular pathway in crops like tomato and rice are revisited. The phenotypes of edits generated across genetic backgrounds were assessed and compared with available resequencing and phenotypic data. The traits evaluated in the current perspective were *de novo* domestication, grain quality, fruit colour, yield-related traits and stress tolerance to check whether the data available give significant leads to address these traits via editing in other crops. The rationale for editing a particular gene lies in the understanding of the diverse alleles generated, and in this perspective we attempt to discern both the advancements made and the bottlenecks encountered.
- **Conclusions** The effectiveness of gene editing relies significantly on the roles of alleles generated in regulating specific genetic pathways. It is essential to conduct functional validation of the targeted allele across multiple distinct genetic backgrounds to ascertain its utility. The influence an allele exerts on a given trait is contingent upon factors like the nature of the trait, the position of the gene within a given pathway, and the genetic background in which it has been/will be tested. This perspective highlights how editing has led to a range of phenotypic variations influenced by the genetic background, with certain lines achieving the desired phenotype alongside pleiotropic effects, whereas others do not manifest the expected phenotype. This challenge may be addressed by prioritizing the identification of the right candidate and specific motifs in the regulatory regions as potential targets rather than directly intervening in coding sequences.

**Key words:** Alleles, genetic background, trait, tomato, rice, phenotype.

### INTRODUCTION

Many agronomic traits are controlled by multiple loci, making it challenging to select plants carrying the desired alleles simultaneously at these loci (Würschum, 2012). In the case of hybrid breeding in cereals, genetic diversity at key loci is typically fixed in inbred lines, which are then crossed to produce homogeneous and vigorous progenies, thereby limiting opportunities for further enhancement of traits through breeding (Longin *et al.*, 2012). The progress of crop improvement has been hastened by employing genome editing technology like CRISPR/Cas9 to introduce targeted genetic mutations into existing elite varieties. However, the efficacy of this tool hinges significantly on the identification of suitable genes, alleles and haplotypes for intended traits. Whole-genome sequencing of various crops alongside the availability of pangenome sequences has

facilitated the identification of potential allelic combinations linked to desirable traits (Tay *et al.*, 2022).

The current perspective underscores both the progress and challenges related to applying CRISPR/Cas9 technology in improving traits, with a particular emphasis on key agronomic attributes in tomato and rice, where substantial editing efforts have been conducted. Our discussion is focused on the influence of alleles and haplotypes within various genetic backgrounds on trait enhancement. Additionally, we conducted SNP analysis for two selected candidates, one associated with grain yield in rice and the other involved in fruit development in tomato. This analysis suggests that gene editing has been specifically directed towards certain alleles within selected genotypes rather than across diverse genetic backgrounds. This raises the question of whether targeting the same allele would

yield similar phenotypes across diverse genetic backgrounds, and several studies have demonstrated that the genetic background determines phenotypic outcomes. The present perspective is organized into four sections: (1) exploring the impact of CRISPR/Cas9 on tomato fruit development, (2) unleashing the potential of CRISPR/Cas9 for improving agronomic traits in rice, (3) identifying bottlenecks and solutions for generating a functional allele, and (4) conclusions. Each section is further divided into relevant subsections that include studies on stress tolerance along with SNP analysis of selected tomato and rice genes incorporated within the corresponding crop sections.

#### EXPLORING THE ROLE OF CRISPR/CAS9 IN UNDERSTANDING TOMATO DEVELOPMENT

CRISPR/Cas9-mediated gene editing has been harnessed in tomato since the advent of this method, resulting in notable progress in elucidating gene functions and facilitating precise breeding opportunities (Brooks *et al.*, 2014). As a proof of concept, initially, two gRNAs were cloned, targeting the second exon of the tomato homologue of *Arabidopsis ARGONAUTE7 (AGO7)* in *S. lycopersicum* ‘M82’ (Brooks *et al.*, 2014). *AGO7* plays a crucial role in the biogenesis of *trans*-acting short interfering RNAs, which are essential for the post-transcriptional silencing of *AUXIN RESPONSE FACTOR (ARF)* genes, ultimately influencing organ polarity (Brooks *et al.*, 2014). Mutant plants with strong alleles of *ago7* exhibited reduced levels of *trans*-acting short interfering RNAs and decreased ARF mRNA degradation. Consequently, the first leaves of these wiry mutants lacked petioles, while the subsequent leaves had no laminae (Brooks *et al.*, 2014). *SHORT-ROOT (SHR)* and *SCARECROW (SCR)* are members of the GRAS (GIBBERELLIC-ACID INSENSITIVE [GAI], REPRESSOR of GAI, and SCR) transcription factor family, which is essential in plant development (Helariutta *et al.*, 2000). In *Arabidopsis*, *SHR* regulates the expression of *SCR* as mutations in the *AtSHR* reduced *SCR* expression, which resulted in the short-root phenotype characterized by impaired stem cell division and disrupted cell patterning (Helariutta *et al.*, 2000). A gRNA targeting the GRAS domain of *SHR* was introduced into tomato (Ron *et al.*, 2014). The resulting mutant roots displayed phenotypes that are consistent with *Arabidopsis shr* mutants (Benfey *et al.*, 1993). This study reinforces that the function of *SHR* is evolutionarily conserved, particularly in regulating its downstream targets and influencing root length in both *Arabidopsis* and tomato. Subsequently, CRISPR/Cas9 has been applied for editing in cultivated and wild tomato genotypes, such as *Solanum lycopersicum* var. *cerasiforme* (Kuroiwa *et al.*, 2023), *S. pimpinellifolium* (Wu *et al.*, 2018; Zsögön *et al.*, 2018) and *S. peruvianum* (Lin *et al.*, 2022). As of March 2025, 546 primary references are available detailing the utilization of CRISPR/Cas technology for gene editing within tomato and related species. In the following subsections on tomato, the progress achieved in fruit morphology, firmness, delayed ripening and stress resilience using CRISPR/Cas mutagenesis is discussed. These studies reveal that implementing CRISPR technology requires careful consideration of gene functions, genetic backgrounds and underlying mechanisms.

#### Advances in tomato fruit morphology using CRISPR/Cas mutagenesis

Several studies were conducted to delineate the evolutionary process of domestication from the probable ancestor, *S. pimpinellifolium*, to modern-day tomato cultivars (Blanca *et al.*, 2015). Numerous domestication traits exhibit Mendelian inheritance patterns (Meyer and Purugganan, 2013), indicating the possibility of reintroducing these traits into a suitable genetic background through CRISPR/Cas9 genome editing. Using multiplex CRISPR/Cas9 editing, six genetic loci associated with pivotal domestication attributes, such as plant architecture (*SELF-PRUNING, SP*), fruit morphology (*OVATE, O*; *FASCIATED, FAS*, and *FRUIT WEIGHT 2.2, FW2.2*), yield (*MULTIFLORA, MULT*) and quality (*LYCOPENE BETA CYCLASE, CycB*) were targeted (Zsögön *et al.*, 2018). A total of six gRNAs, each corresponding to one gene, all regulated by a single CaMV35 promoter, were transformed in *S. pimpinellifolium* (Zsögön *et al.*, 2018). Four genes (*SP, O, FW2.2* and *CycB*) exhibited edited alleles, whereas no successful mutants were obtained in *FAS* or *MULT*. On further analysis, a G→A substitution was found at the gRNA target site in the *S. pimpinellifolium* genome, which might have hindered gRNA binding and the subsequent editing process (Zsögön *et al.*, 2018). Moreover, another factor contributing to the lack of editing in these two genes is that they were initially targeted with a single gRNA. Subsequently, editing was achieved when two gRNAs were employed for each gene, indicating that multiple gRNAs directed at a particular gene increase the likelihood of successful editing. The locus associated with the multilocular fruit phenotype of *FAS* was initially attributed to the downregulation of a *YABBY* transcription factor gene (Cong *et al.*, 2008). However, further studies revealed that *YABBY* is located proximal to *CLV3* on chromosome 11 and the observed phenotype is a result of a 294-kb inversion in the *FAS* locus with indels in the *YABBY* intron and *CLV3* promoter (Xu *et al.*, 2015). Hence, a subsequent round of multiplex editing was performed, targeting different coding regions of *FW2.2, FAS, MULT* and *CycB*. This involved using a total of eight gRNAs, with each gRNA directed at two sites within these four genes, all governed by a single CmYLCV promoter. As a result, two lines with loss-of-function mutations in all four targeted loci were identified (Zsögön *et al.*, 2018). The edited lines exhibited increased fruit size, number and nutritional content compared with the wild progenitor, *S. pimpinellifolium*. These lines also displayed an increase in fruit lycopene accumulation compared with the cultivated tomato *S. lycopersicum*. This study, for the first time, provided comprehensive evidence for the use of multiple gRNAs to achieve editing of candidate genes to enhance yield and improve quality traits in crops. It also proved that the targeted reverse genetic engineering of wild species can facilitate the rapid development of improved crop varieties.

#### Role of CRISPR/Cas9 in enhancing fruit firmness

Fruit firmness is a trait of interest in breeding as it favours storage, transportation and consumer preferences. It is also a complex trait influenced by various factors involving different pathways (Romero and Rose, 2019). In tomato, gibberellins (GAs) regulate fruit growth and firmness (Li *et al.*, 2019).

One key regulator is the *qFISI* QTL (FIRMSKIN), located on chromosome 10's short arm, which controls firmness in wild and cultivated varieties through its underlying gene (Li *et al.*, 2020). The underlying gene, *FISI*, encodes GA2-oxidase, which catabolizes bioactive GA into its inactive form. Knockout of *FISI* results in elevated GA, which can lead to increased fruit firmness. Deletions in *FISI* using CRISPR/Cas9 with two gRNAs targeting the first exon and intron, respectively, resulted in null mutants that exhibited firm fruits without any perturbed plant phenotypes in the wild species *S. pimpinellifolium* and cherry tomato 'TS205'. The *fis1* allele was also preferentially selected during the domestication of tomato and contributed to the enhanced firmness (Li *et al.*, 2020).

#### Harnessing CRISPR/Cas technology to delay tomato fruit ripening

Tomato fruit colour is influenced by the pigmentation of its peel and flesh. Although various colour traits have been integrated into elite tomato varieties through several generations of backcrossing, the cultivation of varieties with different coloured fruits necessitates the incorporation of multiple genetic loci into a single genetic background, which may result in abnormal phenotypes. The shift in colour that transpires as fruits undergo ripening is a consequence of the accumulation of naringenin chalcone (NarCh) and lycopene as well as being due to the degradation of chlorophyll and  $\beta$ -xanthophyll (Naem *et al.*, 2023). *Phytoene Synthase 1* (*PSY1*), *MYB12* and *STAY-GREEN 1* (*SGR1*) are integral to the development of different fruit colours in tomato (Yang *et al.*, 2023a). *PSY1* is a key enzyme in carotenoid biosynthesis, promoting the accumulation of  $\beta$ -carotenoid and lycopene while negatively regulating the biosynthesis of NarCh (Zhu *et al.*, 2018). *MYB12* transcription factor is essential in regulating the production of NarCh, and *SGR1* inhibits chlorophyll degradation during ripening (Zhu *et al.*, 2018).

The CRISPR/Cas9 approach generated a green-fruited triple mutant (*psy1 myb12 sgr1*) from the wild-type red-fruited cultivar 'Ailsa Craig' (Yang *et al.*, 2023a). Single (*psy1*, *myb12*, *sgr1*), double (*psy1 myb12*, *myb12 sgr1* and *psy1 sgr1*) and triple (*psy1 myb12 sgr1*) knockout mutants were produced by targeting two gRNAs to the first exon of *PSY1* and *MYB12*, as well as the third exon of *SGR1*, producing multiple alleles resulting in a range of fruit colours (Yang *et al.*, 2023a). The *psy1* single mutants exhibited reduced levels of lycopene and  $\beta$ -carotene with a pronounced accumulation of NarCh, imparting yellow colour. A knockout mutation of *MYB12* inhibited NarCh accumulation, resulting in pink-coloured fruits. Similarly, knockout of *SGR1* affected lycopene accumulation during ripening, producing brown fruits. This strategy of efficiently targeting a transcription factor gene (*MYB12*) and two carotenoid biosynthetic pathway genes (*PSY1* and *SGR1*) enabled rapid development of transgene-free new tomato varieties with different coloration in a shorter duration and no linkage drag as compared with the conventional backcross breeding technique (Yang *et al.*, 2023a).

NAC is a key family of transcription factors. While NAC4, NAC9 and NAC22 positively regulate carotenoid accumulation, the overexpression of NAC1 is associated with a reduction in the ripening process of tomato (Ma *et al.*, 2014; Zhu *et al.*,

2014). Knockout of *NAC9* by targeting five gRNAs in all three exons (three gRNAs targeting exon 1 and one gRNA in each of exon 2 and exon 3) through CRISPR/Cas9 led to a marked decrease in lycopene, chlorophyll and total carotenoid levels in 'Micro-Tom' edited plants (Feng *et al.*, 2023). *nac9* delays chloroplast-to-chromoplast transformation by modulating key genes such as *PSY1*, *DXS2*, *SGR1* and *CrtR-b2* involved in carotenoid metabolism during tomato ripening (Feng *et al.*, 2023). CRISPR/Cas mutagenesis of *NAC4* was conducted using four gRNAs, all directed at the second exon. The *nac4* mutants demonstrated a delayed ripening process, aligning with the previous finding that *NAC4* is positively involved in fruit ripening (Gao *et al.*, 2021). The tomato genome has four anthocyanin-related MYB transcription factor genes, *ANT1*, *ANT1-like*, *AN2* and *AN2-like/Aft*. While *AN2-like/Aft* influences anthocyanin levels in the fruit, the role of other proteins is unclear. CRISPR/Cas9 was used as a functional validation tool to understand the role of *AN2* in the purple tomato 'Indigo Rose'. Two gRNAs targeting R2 and R3 domains within the second exon produced three *AN2* alleles with fruit phenotypes similar to wild type. This suggests that *AN2* alone is not responsible for anthocyanin accumulation in the fruit of 'Indigo Rose' (Zhi *et al.*, 2020).

Tomato fruit ripening is mainly influenced by factors such as transcriptional regulation of ripening-related genes, ethylene signalling and methylation of regulatory regions associated with ripening (Gao *et al.*, 2020). Ethylene application stimulates ripening; however, mutants lacking functional ethylene biosynthesis/signalling pathways cannot initiate ripening. Also, ethylene does not induce ripening in immature fruits, suggesting the involvement of other developmental cues in both fruit and seed maturation. The functions of tomato ripening genes were often characterized by selecting mutants displaying a ripening phenotype and mapping the genes responsible. Tomato ripening inhibitor (*rin*; Robinson, 1968), non-ripening (*nor*; Tigchelaar, 1973) and colorless non-ripening (*Cnr*; Thompson *et al.*, 1999) are the result of spontaneous mutations in genes that encode transcription factors. These include the MADS-domain ripening inhibitor (MADS-RIN), the NAC transcription factor non-ripening (*NAC-NOR*) and the SQUAMOSA promoter binding protein-like (SPL) colourless non-ripening (SPL-CNR), respectively (Gao *et al.*, 2019, 2020). These were considered master regulators of ripening because the phenotypes of corresponding spontaneous mutants lacked ripening initiation. The expression levels of all these transcription factors significantly increase during ripening, suggesting involvement in the transcriptional regulation of ripening (Wang *et al.*, 2019a). MADS-RIN interacts with the promoters of various genes involved in ripening, ethylene production, aroma development, cell wall softening and pigment synthesis, as well as other transcription factors like *NOR*, *CNR*, *APETALA2a* (*AP2a*) and *FUL1/2* (Bemer *et al.*, 2012; Wang *et al.*, 2019a).

CRISPR/Cas9-induced null mutations in *MADS-RIN* demonstrated a mild ripening phenotype compared with the complete absence of ripening observed in spontaneous *rin* mutants (Wang *et al.*, 2019a). This discrepancy arises from the partial or complete lack of repression of ripening-related genes in CRISPR *RIN* mutants, in contrast to the complete repression seen in spontaneous *rin* mutants (Ito *et al.*, 2017). In spontaneous *rin* mutants, a truncated protein is produced which forms a chimera with the neighbouring MACROCALYX (MC) (Ito

*et al.*, 2017). *MC*, which is regulated by MADS-RIN, plays a significant role in repressing the expression of ripening-related genes in natural *rin* mutants. This also suggests that *RIN* acts in a dominant negative manner whereby the truncated protein interacts with other interlocus proteins and binds to their regulatory regions but without transcriptionally activating them (Wang *et al.*, 2019a). Therefore, targeting such negative regulators, which have a pivotal role in regulation, necessitates the design of gRNA towards the C-terminal, leading to a truncated protein instead of no protein.

Similarly, CRISPR/Cas9 *NAC-NOR* knockout mutants, which utilized two gRNAs to target the first and second exons of *NAC-NOR*, also produced a truncated protein (Gao *et al.*, 2020). However, the resultant mutants displayed a less pronounced phenotype with partial non-ripening traits than spontaneous *nor* mutants, which failed to ripen (Kumar *et al.*, 2018). Likewise, the application of CRISPR/Cas9 technology with four gRNAs targeting the second exon of *SPL-CNR* generated knockouts that displayed a ripening delay of only 2–3 d (Do *et al.*, 2024). This phenotype is also distinct from the non-ripening trait of natural *cnr* mutants. Additionally, ripening-related genes such as *ACS2*, *ACO1*, *PSY*, *PG* and *EXP* were not completely downregulated in *nor* and *cnr* CRISPR/Cas9 lines compared with the original mutants (Giovannoni *et al.*, 2007; Wang *et al.*, 2020a). All three spontaneous mutants of *cin*, *nor* and *cnr* as opposed to their CRISPR mutants were characterized by their inability to produce ethylene, soften the pericarp, or change colour from green to deep red (Wang *et al.*, 2020b). These findings suggest that critical biological processes such as ripening are governed by complex networks of redundant genes, and multiplex editing of transcription factors and key pathway genes in the desired domains could result in specific phenotypic outcomes.

TOMATO AGAMOUS-LIKE 1 (*TAGL1*) is a MADS-box transcription factor that regulates ethylene biosynthesis by binding to the promoter of the gene encoding aminocyclopropane-1-carboxylic acid synthase 2 (*ACS2*), a rate-limiting enzyme in the ethylene biosynthesis pathway (Itkin *et al.*, 2009). Overexpression of *TAGL1* as a chimeric repressor lowered ethylene levels and inhibited the breakdown of chlorophyll while also downregulating ripening-related genes (Itkin *et al.*, 2009). *TAGL1* knockout mutants were generated using CRISPR/Cas9 to reassess the role of *TAGL1* by targeting the first exon with two gRNAs. The three *tagl1* mutants produced truncated proteins with reduced ethylene production, increased firmness and a delay in fruit ripening (Jeon *et al.*, 2024).

In efforts to increase tomato fruit firmness, pectin-degrading enzymes are promising targets. CRISPR/Cas9 was used to induce mutations in *Pectate Lyase (PL)*, *Polygalacturonase 2a (PG2a)* and  $\beta$ -*Galactanase (TBG4)* using a single gRNA to target each gene separately in ‘Ailsa Craig’ (Wang *et al.*, 2019b). These mutants exhibited varied levels of transcripts, with *PG2a* showing a significant decrease in transcript levels. Mutations in *PL* solely led to firmer fruit, while those in *PG2a* and *TBG4* affected only fruit colour and its weight (Wang *et al.*, 2019b), which contrasts with earlier findings on *TBG4* in the ‘Rutgers’ cultivar (Smith *et al.*, 2002), where *TBG4* antisense lines displayed increased fruit firmness. Additionally, reduced *PL* activity has been associated with delayed softening in ‘M82’ (Uluisik *et al.*, 2016) and ‘Micro-Tom’ (Yang *et al.*, 2017),

underscoring the importance of this gene in fruit ripening. The difference in the phenotypes of *TBG4* mutants could be attributed to different genetic backgrounds, or the transcript levels in the CRISPR mutants were not sufficiently reduced to produce a firm fruit phenotype as they were targeted by a single gRNA. This further highlights the importance of multiplex gRNAs in effectively knocking out the function of a candidate gene.

Similar to the roles of *CIN*, *NOR* and *CNR*, whose functions were elucidated based on phenotypes of spontaneous mutants, the roles of other important transcription factors involved in fruit ripening, such as *APETALA2a (AP2a)* and *FRUITFULL (FUL1/TDR4 and FUL2/MBP7)*, have also been reassessed using CRISPR/Cas9 by comparing the CRISPR mutants with their corresponding spontaneous or RNAi mutants (Wang *et al.*, 2019a). *AP2a* belongs to the *APETALA2/ethylene response factor (AP2/ERF)* family. RNAi-mediated silencing of *AP2a* demonstrated its role as a negative regulator of ethylene production while also facilitating carotenoid synthesis and chlorophyll degradation (Karlova *et al.*, 2011). *FUL1* and *FUL2*, which are homologues of MADS-box *FRUITFULL*, interact with MADS-RIN and serve as key regulators in ripening (Fujisawa *et al.*, 2014). Tomato possesses two homologues of the MADS-domain transcription factors *FUL1* and *FUL2*. Two gRNAs were used to target the first exon of *AP2a*. Similarly, two gRNAs for each of the second and third exons of *FUL1* and a single gRNA directed at the first exon of *FUL2*, which is a part of the MADS domain, were used to generate *ful1* and *ful2* single mutants, respectively. Further, the combination of one gRNA for *FUL1* and another for *FUL2* facilitated the production of *ful1 ful2* double mutants (Wang *et al.*, 2019a). Four mutant alleles of *AP2a* were identified, among which two null mutants produced twice the amount of ethylene as the wild type, suggesting that *AP2a* is a negative regulator in both ethylene production and the onset of fruit ripening (Wang *et al.*, 2019a). Both *ful1* and *ful2* single and double (*ful1 ful2*) knockout CRISPR mutants were produced. The *ful1 ful2* double mutants of CRISPR had a phenotype similar to the corresponding double knockdown RNAi lines in which ripening was completely inhibited (Shima *et al.*, 2014), whereas the *ful1* and *ful2* single CRISPR mutants exhibited normal phenotype, indicating that they act redundantly in fruit ripening (Wang *et al.*, 2019a). Tomato carries four *FUL* clade genes, *FUL1*, *FUL2*, *MADS-BOX PROTEIN10 (MBP10)* and *MBP20*. To study the role of *MBP10* and *MBP20* transcription factors in fruit development and ripening, CRISPR double (*ful1 ful2 mbp10 mbp20*), triple (*ful1 ful2 mbp10/mbp20*) and quadruple (*ful1 ful2 mbp10 mbp20*) mutants were generated in ‘Moneyberg’ (Jiang *et al.*, 2022). Phenotypic analysis revealed that the double mutants had traits like *ful2* single mutants, while triple and quadruple mutants resembled *ful1 ful2* double mutants, indicating that *MBP10* and *MBP20* do not contribute to fruit development and ripening in the given condition (Jiang *et al.*, 2022).

#### Revisiting stress tolerance in tomato using CRISPR/Cas9 mutagenesis

The advancement of pathogen-resistant cultivars hinges on the application of dominant resistance *I* genes. However, the durability of resistance provided by a single *R* gene is often compromised, as pathogens can swiftly mutate their

corresponding effectors (Dangl *et al.*, 2013). An effective alternative is the incorporation of multiple *R* genes via gene stacking (Fuchs, 2017). Compared with the conventional breeding approach for *R* gene stacking, the targeted disruption of a single susceptibility (*S*) gene can facilitate the rapid establishment of broad-spectrum and long-lasting disease resistance in crops. Nonetheless, it is important to consider the pleiotropic effects of *S* gene inactivation, as these genes also have developmental roles (Van and Takken, 2014).

The natural mutant of cherry tomato (*S. lycopersicum* var. *cerasiforme*), which is resistant to powdery mildew, originates from an Ecuadorian accession and possesses an *ol-2* allele (Bay *et al.*, 2008). This allele has a 19-bp deletion in the coding region of the *MLO1* gene, leading to a premature stop codon located within the second cytoplasmic loop of the corresponding protein (Bay *et al.*, 2008). It provides a broad-spectrum, recessively inherited resistance to the powdery mildew fungus *Oidium neolyopersici* (Bay *et al.*, 2008). Gene silencing techniques suggest that the powdery mildew resistance associated with *ol-2* is due to the loss of function of *mlo1* (Bay *et al.*, 2008). *MLO1* is an *S* gene essential for the effective colonization of fungi; when it is knocked out the papillae obstruct fungal penetration into epidermal cells, leading to resistance (Lyngkjaer *et al.*, 2000). Sixteen *MLO* genes have been identified, with *MLO1* as the principal contributor to powdery mildew susceptibility in tomato (Yan *et al.*, 2021). Although natural non-transgenic *mlo1* mutants are available in tomato, introducing these alleles into a superior breeding cultivar is time-consuming and laborious. The CRISPR/Cas9 system created a transgene-free *MLO1*-edited new tomato variety, ‘Tomelo’, in the ‘Moneymaker’ background (Nekrasov *et al.*, 2017) in <10 months. This was achieved by targeting the sole exon of *MLO1* with two gRNAs separated by a 42-bp region (Nekrasov *et al.*, 2017). Two of the ten primary transformants displayed homozygous deletions of 48 bp located upstream of the protospacer adjacent motif (PAM) sequence, with no evidence of off-target mutations (Nekrasov *et al.*, 2017). The use of CRISPR/Cas9 has not only generated a beneficial allele but has also resulted in the fast development of the ‘Tomelo’ variety, which exhibits resistance to powdery mildew.

Additionally, the *DOWNY MILDEW RESISTANT 6* (*DMR6*) gene in *Arabidopsis*, which encodes a 2-oxoglutarate Fe(II)-dependent dioxygenase, is involved in defence against bacterial and oomycete pathogens (Van Damme *et al.*, 2008). CRISPR/Cas9 was employed to study the function of another *S* gene, *DMR6-1*, to induce broad-spectrum and long-lasting disease resistance by directing two gRNAs to the second and third exons (Thomazella *et al.*, 2021). Among 61 *T<sub>0</sub>* plants, five carried homozygous deletion alleles that produced a truncated protein with a disrupted *DMR6* active site (Thomazella *et al.*, 2021). These mutants exhibited reduced disease severity when challenged with bacterial pathogens (*Pseudomonas syringae* pv. Tomato, *Xanthomonas gardneri* and *X. perforans*), an oomycete (*Phytophthora capsica*) and powdery mildew (*Pseudoidium neolyopersici*) without pleiotropic effects. The increased resistance to pathogens in the *sldmr6-1* mutants was associated with a salicylic acid-mediated immune response (Thomazella *et al.*, 2021). Four gRNAs targeting three exons of *Defense No Death 1* (*DND1*) were introduced into the tomato ‘Moneymaker’ cultivar (Li *et*

*al.*, 2024). The homozygous edited plants exhibited reduced symptoms of powdery mildew (Li *et al.*, 2024). Prior studies involving RNAi-silenced *DND1* plants indicated a reduction in powdery mildew symptoms; however, the phenotype of these plants was severely compromised (Sun *et al.*, 2017a). Similarly, two CRISPR mutants carrying truncated *DND1* proteins demonstrated resistance to powdery mildew but with negative impacts on plant phenotype. However, one *dnd1* CRISPR mutant with 3- and 6-bp deletions in exons 2 and 4, respectively, exhibited resistance to powdery mildew with minimal pleiotropic effects (Li *et al.*, 2024). The three amino acid deletions induced conformational changes in the *DND1* protein, affecting its function (Li *et al.*, 2024). This study highlights the importance of generating a range of alleles so that a superior allele with minimum pleiotropic effects can be selected. This also helps in understanding the function of the targeted gene, as conformational changes were sufficient to achieve the desired phenotype in this case.

*POWDERY MILDEW RESISTANCE 4* (*PMR4*) is a callose synthase gene that encodes CALLOSE SYNTHASE 12 or GLUCAN SYNTHASE-LIKE 5, which is essential for callose deposition in response to biotic and abiotic stresses (Nishimura *et al.*, 2003). CRISPR/Cas9 knockout mutants of *PMR4* were generated by employing four gRNAs, conferring resistance to powdery mildew in the susceptible tomato cultivar ‘Moneymaker’ (Santillán Martínez *et al.*, 2020). The role of the *PMR4* gene was also validated to induce resistance to *Phytophthora infestans* in ‘San Marzano’ and ‘Oxheart’ (Li *et al.*, 2022). The *PELOTA* (*PELO*) gene, designated as the *ty-5* allele (introgressed from *S. peruvianum*) is located on chromosome 4, spans 7740 bp and encodes a 387-amino acid homologue of an mRNA surveillance factor involved in the ribosome recycling phase of protein synthesis and resistance to Tomato yellow leaf curl virus (TYLCV) (Pramanik *et al.*, 2021). It has three conserved eukaryotic translation termination factor 1 (eRF1) domains. The function of *PELO* has been effectively re-evaluated through CRISPR/Cas9, which involved targeting nine gRNAs within the eRF1-1 domain. Among these, only one gRNA at target site 1 of *PELO* demonstrated successful editing in commercial tomato cultivar ‘BN-86’ (Pramanik *et al.*, 2021). The observed low efficiency of the gRNAs has been linked to the instability of the Cas9–gRNA complex, along with the limited availability of Cas9 when multiplexing gRNAs driven by a single constitutive promoter. Three *pelo* knockouts produced early termination codons, resulting in truncated proteins with loss-of-function alleles. The RNAi *pelo* mutants halted viral replication and resulted in a phenotype resistant to TYLCV (Lapidot *et al.*, 2015). Similarly, the CRISPR *pelo* mutants also exhibited no symptoms of TYLCV and demonstrated a marked decrease in TYLCV copy number. This study suggests that a single promoter can be used to drive the expression of three gRNAs simultaneously and, if one wants to aim for a number more than that, a different multiplexing strategy should be deployed.

*DMR6*, *MLO1*, *DND1* and *PELO* function as negative regulators of disease resistance, whereas the MYB transcription factor gene *MYBS2* has been identified as a positive regulator. CRISPR knockout mutants targeting the first exon of *MYBS2* exhibited diminished resistance to *P. infestans* as evidenced by an increase in the number and size of disease lesions (Liu *et al.*, 2021). This

phenotype was correlated with the downregulation of genes associated with ROS scavenging in *mybs2* mutants, indicating that MYBs are the key transcription factors in mediating resistance. The knockout of negative regulators leads to the development of resistance; consequently, activation or enhancement of the expression levels of positive regulators, which can be achieved by incorporating activators or short enhancer sequences within regulatory regions, promotes disease resistance.

CRISPR/Cas9 is also used to mitigate the impacts of abiotic stresses caused by climate change, ultimately aiming to sustain yields and ensure food security. Knockout of tomato *LBD40* (*LATERAL ORGAN BOUNDARIES DOMAIN 40*), which is a class 2 family of LBD genes, expresses significantly in the roots and fruits, resulting in improved water-holding capacity under drought stress (Liu *et al.*, 2020). The expression of *LBD40* was also induced by PEG, salt and jasmonic acid (JA), and it operates downstream of MYC2, which is the key transcription factor in the JA signal transduction pathway (Liu *et al.*, 2020). Additionally, the knockout of the *ARF4* (*Auxin Response Factor 4*) gene, with reduced expression levels under limited water availability and exposure to ABA, enhanced the ability to recover from salt (ionic) and osmotic stress (Chen *et al.*, 2021). This was coupled with a noticeable leaf-curling phenotype and a significant increase in both root length and density, along with reduced transpiration rates (Bouzroud *et al.*, 2020; Chen *et al.*, 2021). *SELF PRUNING 3C* (*SP3C*) is another key candidate that belongs to the *CETS* (*CENTRORADIALIS*, *TERMINAL FLOWER 1*, *SELF PRUNING*) gene family, governing many traits of agronomic importance (Moreira *et al.*, 2022). CRISPR/Cas9 editing of *SP3C* displayed characteristics associated with drought tolerance, such as long roots and reduced lateral root branching (Moreira *et al.*, 2022). Further investigation into the functions of crucial transcription factors that regulate response to abiotic stress through editing could enhance our capabilities in developing tomato varieties that are resilient to multiple stressors.

#### Single-nucleotide polymorphism analysis of tomato genes to identify potential targets for editing

Although various genes regulating important traits have been identified, the quest for optimal haplotype combinations to produce a superior variety has yet to yield definitive results. SNP analysis was conducted on *Solyc04g040190* (*Cyclin B*; *CycB*), which encodes lycopene  $\beta$ -cyclase (*CycB*), across 39 tomato genotypes catalogued in the Tomato Functional SNP database (<https://plant1.kazusa.or.jp/tomato/>). Variations were detected at three specific nucleotide positions (31103944, 31104692, and 31104807). At position 31103944 the predominant nucleotide is C, with seven genotypes exhibiting replacement with A, including three *S. pimpinellifolium* genotypes, as well as ‘sweet100’, ‘Regina’, *S. lycopersicum* ‘LA925’, ‘M82’ and ‘M82\_MM’. At position 31104692, the G nucleotide is replaced by A in the *S. peruvianum* genotype WIR2020, while at position 31104807 the A is replaced by T in two *S. pennellii* genotypes, ‘LA716’ and ‘TOMJPF00008’. The observed variations in the coding region are synonymous, indicating that there are no changes in the corresponding protein sequences. This variation may be of relevance if these regions are targeted for gRNA design in the above-mentioned genotypes.

SNP analysis of *Solyc05g050010*, encoding aminocyclopropane-1-carboxylic acid synthase, is implicated in ethylene production during the ripening of tomato fruits, identified variations at five positions (59035173, 59035255, 59035373, 59035974, 59037030 and 59037225). At each of these positions, a deletion or an addition of a nucleotide was observed. For example, at positions 59035173 and 59035255 a T nucleotide addition was detected in 17 genotypes, while the others exhibited a deletion. Likewise, at locus 59035373 a G addition was present in 12 genotypes, with the remaining genotypes showing a deletion of this nucleotide.

The function of *Solyc04g040190* and *Solyc05g050010* was studied through CRISPR/Cas (Zsögön *et al.*, 2018; Hu *et al.*, 2019). However, it is unclear whether targeting the same sites in other genotypes will produce similar phenotypic outcomes. The SNP analysis of *Solyc04g040190* and *Solyc05g050010* was aimed at identifying haplotypes associated with the two candidate genes and detecting alterations in protein sequences resulting from nucleotide variations across various tomato genotypes. Because we could not identify any amino acid variations for both candidates, an alternative strategy for selecting a beneficial allele is to generate a range of CRISPR alleles followed by phenotypic assessment across different genetic backgrounds. Supplementary Data Table S2 presents the SNP analysis for *Solyc04g040190* and *Solyc05g050010* across a cohort of 39 tomato genotypes (names of tomato genotypes are available in Supplementary Data Table S1). Another effective approach involves targeting the upstream regulatory regions of these genes by introducing silencer elements, ensuring that the core promoter is not disturbed. This strategy would facilitate the downregulation of gene expression rather than complete gene knockout. This approach could produce the desired phenotype with minimal pleiotropic effects.

#### UNLEASHING THE POTENTIAL OF CRISPR/CAS9 FOR IMPROVING AGRONOMIC TRAITS IN RICE

Rice, with its smaller genome size among cereals, is an ideal crop to elucidate the functions of candidate genes and alleles for functional genomic studies. It offers advantages such as high genetic transformation efficiency, easy access to genetic resources and strong genomic synteny with other cereals. Also, the rice genome showcases a significant number of potential PAM sites (1 in 10 bp) (Miao *et al.*, 2013), thereby enabling the swift utilization of CRISPR technology for editing potential candidates within the rice genome. All these factors make rice a valuable model crop for investigating multiple candidates for genome modification using the CRISPR/Cas9 system. The following subsections on rice detail the advancements in improving yield, grain quality and stress tolerance by applying CRISPR/Cas9. Some of the studies cited employed CRISPR/Cas directly to introduce the desired phenotype, while others revisited and analysed previous findings using this tool.

##### Enhancing rice productivity

Enhancing rice production and productivity is important due to the reliance of a large proportion of the global population

on rice as their primary energy source. More than 2300 genes associated with various development and stress-related traits have been functionally validated in rice. Furthermore, the re-sequencing of diverse germplasm has led to the identification of allelic variations related to traits of interest, which can be applied in crop improvement programmes (Varshney *et al.*, 2018; Rai and Tyagi, 2022). Yield is a complex trait and is governed by multiple genes and QTL interactions (Shen *et al.*, 2018). In rice, enhanced yield could be due to four key subtraits: the number of effective tillers or panicles per plant, the number of grains per panicle, the percentage of filled grains, and the weight of the grains.

*GRAIN SIZE3 (GS3)* and *Grain number 1a/Cytokinin Oxidase2 (Gn1a/CKX2)* were the first loci to be fine-mapped for grain size (Fan *et al.*, 2006) and grain number (Ashikari *et al.*, 2005), respectively. *GS3* is a major QTL located in the pericentromeric region of chromosome 3. Association studies have shown that C→A mutation in the second exon of *GS3* (A allele) is linked to increased grain length in *Oryza sativa*, this mutation is absent in other species (Fan *et al.*, 2006). *Gn1a/OsCKX2* modulates meristem activity by influencing cytokinin levels; its inactivation leads to heightened cytokinin synthesis, promoting cell proliferation (Li *et al.*, 2013). To investigate whether targeted alleles for these two key genes can be generated by CRISPR technology, both *GS3* and *Gn1a* were simultaneously targeted using a single CRISPR vector with gRNAs directed at the start codon for both the genes in five *japonica* cultivars, ‘N9108’, ‘W27’, ‘Y4227’, ‘Z22’ and ‘Z88’. Both *gs3* single and *gs3 gn1a* double mutants were generated across all genetic backgrounds tested. The grain length of *gs3* and *gs3 gn1a* mutants was increased, with *gs3 gn1a* mutants having a higher grain number. Seven out of ten homozygous mutants displayed a decrease in the number of effective tillers (Shen *et al.*, 2018). This study revealed that the same allele can lead to different phenotypes in different genetic backgrounds. In all five genotypes, similar insertions (G, T or A) were identified at the fourth nucleotide position from the 3’ end of target gRNA in *GS3*. Three genotypes carried additional deletions of 2–58 bp length (Shen *et al.*, 2018). The phenotypic variations can be attributed not only to different genetic backgrounds but also to how the efficacy of the same gRNAs might be influenced by other variations if present within the targeted gene. When the desired phenotype fails to manifest by targeting the coding regions for a complex trait, an effective approach could be to identify and target regulatory motifs such as silencers, activators or signal-responsive elements in the *cis* regions. If these elements are absent in the regulatory regions, their introduction could facilitate the modulation of gene expression to obtain the intended phenotype.

The three genes *GS3*, *GN1a* and *GRAIN WIDTH* and *WEIGHT2 (GW2)*, which are the negative regulators of grain size, number and grain width and weight, respectively, were also simultaneously targeted in rice (Zhou *et al.*, 2019). The first exon of *GN1a*, the second exon of *GS3* and the fourth exon of *GW2* were targeted in the three *japonica* cultivars ‘J809’, ‘L237’ and ‘CNXJ’, resulting in single, double and triple mutants. For each cultivar, 15  $T_0$  plants were selected for genotyping. In the ‘J809’ and ‘L237’ genetic backgrounds, the grain sizes of the *gs3* and *gw2* single mutants were larger, a trait observed in the double and triple mutants (Zhou *et al.*, 2019). The triple

mutants in these backgrounds exhibited a significantly greater number of flowers per panicle, panicle length, and grain length, width and weight. The triple mutants on the CNXJ background demonstrated enhanced panicle length, grain length and grain width, although the plants were semi-dwarf (Zhou *et al.*, 2019). This study demonstrated the feasibility of targeting multiple genes for yield increase and also demonstrated that the genetic interactions due to the alleles generated are dependent on the genetic background as well.

Membrane-localized amino acid transporters facilitate the transport of amino acids from the rhizosphere to the seed. In rice, at least 16 *OsAAPs* have been identified and characterized (Taylor *et al.*, 2015). Some of these increase tillering and overall yield when overexpressed, while others act as negative regulators of yield. For example, *OsAAP1*, *OsAAP4*, *OsAAP6* and *OsAAP11* positively regulate tillering, panicle number and yield, whereas *OsAAP3*, *OsAAP5* and *OsAAP11* function as negative regulators (Peng *et al.*, 2014; Taylor *et al.*, 2015). The function of *OsAAP11* (Os11g0195600) was targeted through CRISPR/Cas9 in the three *japonica* cultivars ‘WYG30’, ‘NG9108’ and ‘YG158’. The first exon of *OsAAP11* was targeted using a gRNA, generating two homozygous mutations in each genetic background. These mutants exhibited varying agronomic traits across different genetic backgrounds (Yang *et al.*, 2023b). A mutant in ‘WYG30’ showed a significant increase in grain length, while the ‘NG9108’ mutant displayed a decrease in plant height, grain length and hundred-grain weight. Different CRISPR alleles within the same genetic background also produced varied phenotypes; for example, the ‘YG158-1’ mutant showed a significant reduction in plant height, while the ‘YG158-2’ mutant showed a significant increase in grain length and width (Yang *et al.*, 2023b).

Similar to *gs3 gn1a* double mutants, the *OsAAP* CRISPR mutants also displayed varying phenotypes depending on the genetic background, further supporting the hypothesis that genotype largely influences gene editing outcomes. Similar to *OsAAP11*, the role of *OsAAP3* was also evaluated through CRISPR/Cas by targeting gRNAs to the sixth exon in *japonica* ‘ZH11’ and ‘KY131’ (Lu *et al.*, 2018). This resulted in eight mutants with a range of base deletions, two having a single base insertion. Biomass of straw and grain yield significantly increased in CRISPR mutants of both genotypes (Lu *et al.*, 2018). *OsAAP3* plays a crucial role in promoting the elongation of outgrowth buds and increasing the number of tillers, thereby influencing grain yield through the regulation of amino acid concentrations, including Lys, Arg, His, Asp, Ala, Gln, Gly, Thr and Tyr. A negative correlation was observed between the expression level of *OsAAP3* and the number of tillers, indicating that *japonica* accessions, characterized by higher *OsAAP3* expression in the culm, also exhibited a reduced number of tillers (Lu *et al.*, 2018). In *OsAAP3* high-expression lines, these amino acids accumulate, suppressing axillary bud formation and tillering (Lu *et al.*, 2018). Although *OsAAP3* has been studied in *japonica* cultivars, the phenotypic implications in *indica* varieties are yet to be explored. It is necessary to assess whether modifying the same locus in *indica* will yield a phenotype comparable to that observed in *japonica*.

Grain width is an important trait for yield and significantly affects grain quality. The major QTL associated with grain width

and weight (*GW2*), located on the second chromosome, encodes a RING-type E3 ubiquitin ligase in rice (Song *et al.*, 2007). Loss of function of *GW2* results in broad and heavy grains due to enhanced cell proliferation in the hulls, ultimately contributing to increased grain yield (Li *et al.*, 2016). *OsZIP47* is a negative regulator of grain width and weight, whereas *WIDE GRAIN 1 (WG1)*, which encodes a glutaredoxin (GRX8), acts as a positive regulator. A working model is proposed to explain the regulation of *GW2*, *WG1* and *bZIP47* in grain size control in rice (Hao *et al.*, 2021). Growth signals trigger the activation of *WG1*, which binds to *bZIP47*, repressing its transcriptional activation by recruiting the transcriptional co-repressor *ASP1 (aberrant spikelet and panicle1)*, which encodes a TPL/TPR (TOPLESS)-related protein. The expression of *bZIP47*-targeted genes is, therefore, suppressed, facilitating cell proliferation. When signals are withdrawn, *GW2* mediates the ubiquitination of *WG1*, leading to its degradation and removing the inhibitory effect of *WG1-ASP1* on *bZIP47* transcriptional activation activity. Consequently, *bZIP47*-targeted genes associated with hormone signalling, carbohydrate metabolism and the cell cycle are upregulated, restricting grain growth (Hao *et al.*, 2021).

The role of *GW2* was evaluated in rice by targeting a gRNA towards the fourth exon within the RING/U-box domain (Achary *et al.*, 2021). Among 189  $T_1$  plants analysed, only two mutations were identified, a single-nucleotide insertion of C and a single-nucleotide deletion of A at the fourth nucleotide position from the 3' terminus of gRNA. Both the mutants exhibited a significantly enhanced aleurone layer highlighting the role of *GW2* in regulating aleurone layer morphology and grain nutritional quality (Achary *et al.*, 2021). A natural mutant, *KEMS39*, was identified in the Koshihikari background that exhibits large grain size and increased yield with improved resistance to lodging. This mutant harbours a G→A polymorphism at the 3' splicing site of the sixth intron of the *GW2*, resulting in the deletion of 67 bp from exon 7. Similar phenotypes were observed when the 3' splice site of the sixth intron was edited using CRISPR/Cas9 (Yamaguchi *et al.*, 2020).

CRISPR/Cas9 for multiplex gene mutagenesis was employed to enhance grain weight in 'LH422' by targeting the *GW2*, *Grain Width 5 (GW5)* and *Thousand-Grain Weight 6 (TGW6)*; all three genes are identified as negative regulators of grain weight. The gRNA target sites for *TGW6* and *GW2* were selected from the first and fourth exons, respectively, while the target site for *GW5* was located within the first exon (Xu *et al.*, 2016). These three gRNAs were integrated into a single vector and cloned as different expression cassettes. Among the 21  $T_0$  transformed lines, 20 were identified as triple mutants (*gw2 gw5t gw6*), while one was a double mutant (*gw5 gw6*). The predominant mutations observed in *GW2* and *TGW6* were short (1–2 bp) indels, which produced truncated proteins (Xu *et al.*, 2016). The mutations in *GW5* were characterized by larger deletions (between 189 and 209 bp). The grain size of the homozygous *gw5t gw6* and *gw2 gw5t gw6*  $T_1$  mutants was significantly greater than that of the wild-type 'LH422'. The grain size and thousand-grain weight of triple mutants surpassed those of the double mutant, indicating that *GW2* functions independently (Xu *et al.*, 2016). This study suggests that pyramiding null mutations in key genes through CRISPR/Cas9 can lead to a marked enhancement in rice grain weight. CRISPR/Cas9 was

also used to enhance the yield by targeting pyruvate enzymes and cell cycle proteins such as *OsSPL16/qGW8 (Os08g41940)* (Usman *et al.*, 2021). Two gRNAs, each designed to target the first exon of *OsSPL16* on opposing strands, were employed to edit *OsSPL16*, resulting in mutant phenotypes that exhibited enhanced grain width and increased thousand-grain weight relative to the wild type.

#### Role of CRISPR/Cas9 in improving rice grain quality

The application of genome-editing techniques for enhancing rice grain quality attributes has also made significant progress. Multiple genes associated with grain quality traits in rice were identified and validated. Amylose content (AC), which indicates the percentage of grain starch, determines the cooking and eating quality of rice. This trait is governed by a single dominant *Waxy* gene (*Wx*, Os06g04200) encoding a granule-bound starch synthase that facilitates the synthesis of amylose in the endosperm (Wang *et al.*, 1995). Two primary *Wx* alleles with a G/T polymorphism are identified: *Wxa*, found in *indica* cultivars, and *Wxb*, present in *japonica* cultivars (Wang *et al.*, 1995). This natural variation leads to differential splicing of *Waxy*, resulting in varying mRNA stability. Consequently, *Wxa* yields higher levels of mRNA and protein than those produced by *Wxb* (Wang *et al.*, 1995). The expression of *Waxy* is directly related to AC, indicating its function as a positive regulator, thus making it possible to modify the AC of rice. The *Waxy* gene was targeted using CRISPR/Cas9, successfully transforming two non-glutinous rice cultivars, '9522' and 'XS134', into glutinous varieties without pleiotropic effects (Zhang *et al.*, 2018). A single gRNA was employed to target the first of six exons, leading to frameshift mutations within the coding region that produced non-functional proteins. In both cultivars, the mutants exhibited no significant differences in plant height, grain count per panicle, panicle count per plant and overall yield from the wild type, suggesting that *Waxy* does not influence key agronomic traits in rice. However, the mutant grains displayed a 'waxy' phenotype with a white and opaque appearance, in contrast to the typical 'non-waxy' translucent look of the wild-type seeds (Zhang *et al.*, 2018). In these mutants, the level of AC was not significantly decreased.

When the promoter of *Wx* was targeted, edited plants with substantial reductions in AC levels within the endosperm were obtained (Huang *et al.*, 2020). Seven target sites were identified in the *Wx* promoter, six sites (S1–S6) located within the predicted *cis* motifs and one (S7) in the core promoter region near the TATA box. The gRNAs designed for pairs of target sites, specifically S1 and S2, S2 and S3, S3 and S4, and S5 and S6, were incorporated into one CRISPR/Cas9 vector, while the gRNA for S7 was cloned separately (Huang *et al.*, 2020). Independent transformation of these two constructs resulted in the generation of 49 plants exhibiting targeted mutations. Although all the edited plants displayed variations in AC levels, the six homozygous plants edited at site S7 demonstrated a marked AC reduction. All these six novel *wx* alleles obtained by editing the region near the TATA box resulted in the downregulation of *Wx* expression and fine-tuning of grain AC (Huang *et al.*, 2020). These findings demonstrate that manipulating specific sites in regulatory regions can effectively produce the desired phenotype. Contrarily, the targeted knockout of the *SBEIIb*

gene, encoding one of the starch-branching enzyme isoforms, has resulted in the development of high-amylose rice, which also holds promise as a beneficial dietary option (Sun *et al.*, 2017b). The third exon of *SBEIIb* was targeted with a gRNA producing mutations ranging from small indels to large deletions. Knockout of *SBEIIb* resulted in a reduced ratio of branch points within the amylopectin fraction, accompanied by an increase in AC (Sun *et al.*, 2017b). However, the increase in AC was more pronounced in transgenic plants expressing antisense RNAs for both *SBEI* and *SBEIIb* (Zhu *et al.*, 2012), suggesting that targeting both isoforms simultaneously could significantly enhance AC rather than focusing on a single gene.

Carotenoids, which are an important source of vitamin A, are not accumulated in the rice endosperm due to the lack of their targeting or the absence of a biosynthetic pathway in endosperm (Zhao *et al.*, 2020). To enhance  $\beta$ -carotene accumulation in rice endosperm, CRISPR/Cas was employed by targeting the knocking out of five genes of the carotene degradation pathway independently (Yang *et al.*, 2017). Five gRNAs, each targeting the second exon of *OsCYP97A4* and *OsCCD7*, the third exon of *OsDSM2* and the sole exon of *OsCCD4a* and *OsCCD4b*, were cloned into separate CRISPR/Cas9 vectors (Yang *et al.*, 2017). The resulting knockout mutants did not show any adverse phenotype except a few *ccd7* mutants, which displayed dwarfism and extensive tillering. The knockout mutants for all five genes exhibited no carotenoid accumulation in the endosperm up to the  $T_1$  generation (Yang *et al.*, 2017). These findings hint that targeting multiple catabolic genes simultaneously, either by multiplexing the gRNAs in a single vector or by crossing plants that have been developed by targeting individual genes, could enhance carotenoid accumulation in rice endosperm, which can be part of future studies for improving this quality trait. This study further highlights the importance of candidate gene selection and the application of multiplexing techniques in achieving the desired phenotype for traits of interest.

Grain chalkiness is defined by small, loosely organized starch granules in the endosperm that produce a chalky or white opaque appearance (Gann *et al.*, 2021). This trait is attributed to disturbances in starch biosynthesis within the rice endosperm, leading to increased grain breakage during milling and affecting palatability. The identification and characterization of potential genes have yielded plants that display a reduction in chalkiness. CRISPR/Cas9 was employed to target coding regions of *Early heading date 1 (EHD1)* with two gRNAs both directed at the third exon in two *japonica* cultivars, ‘Jiyuanxiang 1’ and ‘Yinongxiang 12’ (Song *et al.*, 2024). *Ehd1* spans a 16-kb genomic region on chromosome 10, encoding a 341-amino acid B-type response regulator associated with the heading date (Doi *et al.*, 2004). It has a receptor domain at the N-terminus and a GARP (Golden2, Arabidopsis RESPONSE REGULATOR [ARR]), and *Chlamydomonas* regulatory domain involved in the acclimatization response to phosphorus starvation [Psr1]) DNA-binding motif situated in the middle (Doi *et al.*, 2004). The analysis of the target regions in *ehd1* CRISPR mutants across two genotypes indicated mutation rates of 0 and 47.6 % at the gRNA1 and gRNA2 sites in ‘Yinongxiang 12’, while ‘Jiyuanxiang 1’ exhibited rates of 7.9 and 34.2 %, respectively. These findings demonstrate that editing efficiency varies between the two cultivars, underscoring the impact of different

genetic backgrounds on gene editing for a complex trait like heading date. The CRISPR mutants of both genotypes displayed a marked enhancement in average plant height, number of grains per panicle, and the number of tillers per plant relative to the wild type. The mutants also showed decreased chalkiness, delay in the heading date and improved growth in low-latitude environments.

*Heading date 1 (Hd1)*, *Grain number, plant height and heading date7 (Ghd7)* and *Days to heading 8 (DTH8)* are three major genes acting upstream of *Ehd1* (Yano *et al.*, 2000; Xue *et al.*, 2008; Wei *et al.*, 2010). The knockout mutants of these genes showed an early heading phenotype with adverse effects on agronomic traits (Zong *et al.*, 2024). *Ghd7* has 14 distinct haplotypes in the 3K rice genome panel (Abbai *et al.*, 2019). Among the 1287 accessions, *Ghd7*-H14 was present in the majority, while *Ghd7*-H13 was identified in only 10 accessions. *Ghd7*-H8 was one of the early-flowering haplotypes, in addition to *Ghd7*-H7 and *Ghd7*-H10. Identification of superior haplotypes and the backgrounds in which they work is a strategy that can be adopted when editing for traits for which such information is available. Pleiotropic effects can also be mitigated by targeting the specific regulatory elements as seen in several instances discussed previously. Eight gRNAs driven by a single promoter were designed to target specific regions within the 2-kb promoter regions of *Hd1*, *Ghd7* and *DTH8* separately in the ‘Ningjing8’ (‘NJ8’) cultivar (Zhou *et al.*, 2024). The mutants produced a spectrum of alleles, including SNPs, small indels and large deletions in the *cis* regions. Field trials identified the desired alleles linked to optimal yield, resulting in the broader dissemination of the elite cultivar ‘NJ8’ (Zhou *et al.*, 2024).

For grain chalkiness, *Chalk50*, encoding a 770-amino acid vacuolar H<sup>+</sup> translocating pyrophosphatase (VPPase), has also been identified (Li *et al.*, 2014). Increased expression levels of VPPase, commonly found in *indica* cultivars, are associated with elevated grain chalkiness, which is correlated to the presence of two *cis* elements, the RY/G-box and the CACT tetranucleotide (Li *et al.*, 2014). Breeding for low-chalk varieties is complicated as *Chalk5* is also linked with other QTLs that determine plant yield (Li *et al.*, 2014). To assess the role of *Chalk5* in chalkiness, CRISPR/Cas mutagenesis was performed, targeting promoters with two gRNAs aimed at two sites between -602 and -351 (Gann *et al.*, 2023). Out of 13  $T_0$  plants, 8 displayed identical SNPs that disrupted the GATA element, leading to the downregulation of VPP5 and accumulation of inorganic pyrophosphate within grains. This promoted the formation of large, densely packed granules with minimum air spaces, resulting in the development of translucent grains (Gann *et al.*, 2023). These findings illustrate the importance of targeting specific *cis* elements to modulate gene expression rather than directly altering the coding regions, especially for pleiotropic genes.

#### Enhancing climate resilience in rice

Multiplex editing is promising in inducing broad-spectrum resistance to many pathogens. Knockout mutations in three broad-spectrum blast-resistant genes, *Bsr-d1*, *Pi21* and *ERF922*, in the *indica* thermosensitive genic male-sterile line Longke638S using CRISPR/Cas developed a cultivar that is resistant to both blast and bacterial blight (Zhou *et al.*, 2022).

*Bsr-d1*, located on chromosome 3, encodes a C2H2-type transcription factor that modulates the expression of peroxidase genes, influencing the immune response to *Magnaporthe oryzae*. An SNP in the promoter of the *bsr-d1* gene diminishes its expression due to interaction with a repressive MYB transcription factor (Zhu et al., 2020). *Pi21*, present on chromosome 4, encodes a proline-rich protein with a heavy metal-binding domain and potential protein–protein interaction domain (Fukuoka et al., 2009). *ERF922* encodes a nuclear-localized APETELA2/ethylene response family transcription factor, which is activated by phytohormones, pathogens and abiotic stimuli (Liu et al., 2012). *ERF922* also plays a crucial role in the interaction between biotic and abiotic stress signalling pathways by regulating ABA levels (Liu et al., 2012). The gRNA for *Pi21* was targeted to its second exon, *ERF922* gRNA was aimed towards the first exon, and the gRNA for *Bsr-d1* was directed at its sole exon. These three gRNAs were cloned under distinct promoters and integrated into a single CRISPR vector, generating single and triple mutants without any double mutants (Zhou et al., 2022). All the mutants exhibited enhanced resistance to rice blast, with the *erf922* mutants demonstrating the highest blast resistance equivalent to that of the triple mutants without a compromise in key agronomic traits. This resistance is linked to the upregulation of genes associated with the salicylic acid and methyl jasmonate signalling pathways (Zhou et al., 2022).

The use of CRISPR/Cas9 to target the *Ethylene Response Factor* (*OsERF922*) has provided valuable insights. The findings indicated a noteworthy reduction in the number of blast lesions formed following pathogen infection in all the mutants. Interestingly, no significant differences in agronomic traits were noticed (Wang et al., 2016). Similarly, knockout of *Bsrk1* (*broad spectrum resistance k1*), an RNA-binding protein, conferred rice resistance against *Magnaporthe oryzae* and *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) while maintaining productivity levels (Zhou et al., 2018). *Bsrk1* encodes a tetratricopeptide repeat-containing protein, which binds to mRNAs of multiple *Phenylalanine Ammonia-Lyases* (*OsPAL1-7*) and facilitates their degradation. CRISPR/Cas9-mediated knockout of *bsrk1* leads to the accumulation of *OsPAL1-7* mRNAs. This finding was further reinforced by the overexpression of *OsPAL1* conferring resistance to *M. oryzae* (Zhou et al., 2018).

The *Sugars Will Eventually Be Exported Transporter* (*SWEET*) gene family facilitates sugar transport essential for the growth and development of plants. These genes are exploited by pathogens to obtain nutrients, increasing the susceptibility of plants to diseases (Gupta, 2020). *OsSWEET11*, *13* and *14* are critical susceptibility factors for bacterial leaf blight (BLB) (Yuan and Wang, 2013). Knockout mutants of *OsSWEET13* were generated by targeting its coding region through the CRISPR/Cas system. Two null mutants were produced, one carrying an 11-bp deletion and another with a 4-bp deletion at the target sites. Both mutations effectively disrupted the coding sequence of *OsSWEET13*. The mutants did not exhibit any pleiotropic phenotype but demonstrated enhanced resistance to *Xoo*. The *Xoo* pathogen is known to secrete one or more of six transcription-activator-like effectors (TALEs), which interact with specific promoter sequences referred to as effector-binding elements (EBEs), leading to the activation of sucrose transporter genes like *OsSWEET11*, *13* and *14* (Oliva et al., 2019). The resistant cultivars of rice

were found to have SNPs within the EBEs of the *SWEET* promoters (Oliva et al., 2019). For example, many *indica* varieties possess a *SWEET13* allele containing four adenines in the EBE, rendering them susceptible to pathogenic strains. A multiplex editing approach using gRNAs aimed at the EBEs of *OsSWEET11*, *OsSWEET13* and *OsSWEET14* was developed to systematically disrupt the induction of *SWEET* genes across all six TALE EBEs. This strategy resulted in Kitaake rice being resistant to all known strains of *Xoo* (Oliva et al., 2019).

#### SNP analysis of selected rice genes to identify potential targets for editing

Rice *SPL16* (*Os08g41940*) and *AAP3* (*Os11g09020*) were selected for SNP analysis using the Rice SNP-seek Database (<https://snp-seek.irri.org/>) due to their involvement in improving rice yield and their characterization through CRISPR/Cas9 (Lu et al., 2018; Usman et al., 2021). *OsSPL16* functions as an SBP-domain transcription factor that influences grain width by directly interacting with the promoter of *GW7*, thereby repressing its expression (Wang et al., 2015). *OsAAP3* is an amino acid permease that plays a crucial role in determining yield-related traits in rice, such as the number of tillers. Three major haplotypes of *Os08g41940* were identified through the analysis of 36 SNPs within the coding and UTR regions across 3024 accessions belonging to 12 subpopulations (Supplementary Data Table S3). Within these subpopulations, five are classified as the *indica* group (ind1B, indx, ind2, ind3 and ind1A) while four are categorized as *japonica* (japx, temperate, tropical and subtropical). Additionally, there is one aromatic, one Australian, and one admixture genotype. Within a total of 3024 accessions, 523 (17 %) are classified under haplotype 1, 1637 (54 %) are haplotype 2 and 864 (28 %) are identified as haplotype 3. Out of the 36 SNPs identified from 3024 genotypes, 6 were identified as non-synonymous or missense, resulting in alterations to amino acids. *Os08g41940* has three exons separated by two introns. The second and the third exons contain one and five missense SNPs, respectively, whereas the first exon lacks any annotated missense SNPs (Fig. 1A). However, none of these SNPs generated a stop codon that would yield a truncated protein. Given that the first exon lacks any reported missense SNPs and is conserved across 3024 accessions, this region can be targeted for gRNA design to produce knockout mutants across multiple genetic backgrounds. This will enhance our understanding of the regulatory function of *Os08g41940* in yield and other agronomic traits. Additionally, seven SNPs leading to three haplotypes were identified in the 1-kb upstream region of *Os08g41940* (Supplementary Data Table S3).

Similarly, three haplotypes were identified by examining 30 SNPs in the coding and UTR regions of *Os11g09020* across 3024 accessions from 12 subpopulations (Supplementary Data Table S3). Out of 3024 accessions, 1512 (50 %) are designated as haplotype 1, 95 (3 %) are classified as haplotype 2 and 1417 (47 %) are recognized as haplotype 3 (Fig. 1B). Among the 30 SNPs, 6 were non-synonymous or missense, with 3 located in the first exon, 1 in the second exon, and 2 in the third exon (Fig. 1B). These variations led to modifications in the amino acid sequence without producing a truncated protein. The conserved regions in the three exons can be targeted using multiple

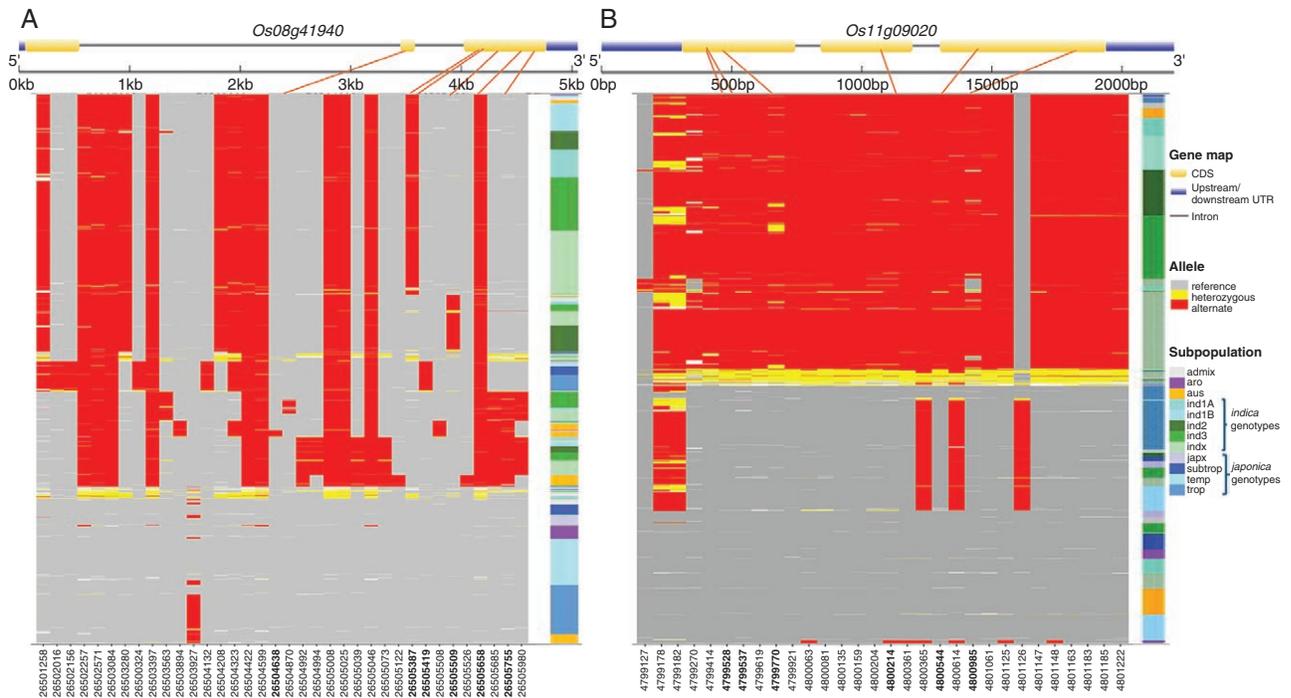


FIG. 1. SNP patterns in the grain quality-related genes (A) *Os08g41940* (*OsSPL16*) and (B) *Os11g09020* (*OsAAP3*) across the 3024 rice genomes. The gene model is represented at the top. The blue and yellow boxes correspond to the UTRs and exons, respectively, and the horizontal line represents the introns. A scale bar to represent the approximate size is provided below the model. The inclined red lines connect the missense SNPs (the positions in nucleotides indicated in bold at the bottom) to the corresponding position in the gene. A snapshot of alleles across 3024 genotypes is shown below the scale bar with vertical coloured bars on the right indicating the subpopulations. The grey, yellow and red horizontal bars indicate reference, heterozygous and alternative alleles, respectively, and white bars denote missing information.

gRNAs to generate potential knockout mutants of this gene. Additionally, within the 1-kb upstream region of *Os11g09020*, three SNPs were identified and categorized into two distinct haplotypes (Supplementary Data Table S3). Haplotype 1 is the most prevalent, accounting for 75 % of the subpopulation, and is characterized by the presence of A, G and T nucleotides at chr11-4799178, 4799182 and 4799270 positions, respectively. The other minor group has G, A and C at these respective locations.

Furthermore, a PLACE analysis (<https://www.dna.affrc.go.jp/PLACE/?action=newplace>) was carried out to analyse whether alterations in *cis* elements correspond to nucleotide variations found in the promoter of *Os11g09020*. The A→G and G→A conversions at positions 4799178 and 4799182 did not alter the existing motif. Both the variations formed DOFCOREZM, a motif required for the binding of zinc finger proteins. The T→C variation at chr11-4799270 resulted in the insertion of NODCON2GM, associated with nodule-specific genes and OSE2ROOTNODULE, an organ-specific motif. The PLACE analysis did not identify any reported silencer or activator at the sites of nucleotide variations, which could have facilitated the modulation of the corresponding gene expression by targeting them. Sometimes, knocking out a gene does not lead to any viable mutants. An effective alternative is to target the upstream regulatory regions by introducing silencer elements or employing multiple gRNAs at different positions, as exemplified by the rice *Wx* promoter (Huang et al., 2020). This could facilitate the downregulation of the gene with minimal pleiotropic effects.

#### IDENTIFYING BOTTLENECKS AND SOLUTIONS FOR GENERATING FUNCTIONAL ALLELES

CRISPR/Cas9 mutagenesis is a highly effective strategy for generating new beneficial alleles to enhance the desirable traits in breeding programmes. Still, the challenge of creating desirable alleles in diverse genetic backgrounds exists. CRISPR/Cas9 studies on tomato fruit development revealed that CRISPR mutants may produce undesirable phenotypes, highlighting the importance of genetic backgrounds and careful selection of target gene and target region within the gene. A classic example is the transcription factor genes *AP2a*, *CIN*, *NOR* and *CNR*, which are important in the ripening process of tomato. Although the role of *AP2a* has been successfully revisited, spontaneous mutants of *CIN*, *NOR* and *CNR* displayed a marked firm fruit phenotype, in contrast to the CRISPR mutants, which did not manifest similar traits. Similarly, antisense *TBG4* mutants of ‘Rutgers’ exhibited enhanced fruit firmness, while the corresponding CRISPR mutants of ‘Ailsa Craig’ did not influence fruit softening, highlighting that the same allele may not induce a similar phenotype in different backgrounds. Likewise, rice CRISPR mutants that target the *AAP11* gene also presented different phenotypes influenced by different genetic backgrounds (Yang et al., 2023b). The WYG30 mutants were characterized by a significant increase in grain length, in contrast to the NG9108 lines, which showed a decline in plant height, grain length and thousand-grain weight. The observed phenotypic variations can be linked to factors like different genetic backgrounds or the insufficient expression levels

of the targeted genes in the CRISPR mutants. Therefore, the increasing prevalence of the CRISPR/Cas9 technique necessitates careful consideration of target genes, target regions and genetic backgrounds. The strategic targeting of specific domains in coding regions or *cis*-regulatory motifs, along with the introduction of silencers/activators, allows the generation of diverse alleles with varying gene expression patterns. This facilitates the selection of desirable alleles without lethal or perturbed plant phenotypes.

## CONCLUSIONS

Considerable advancements have been made in the domestication traits of tomato, as well as in the yield and grain quality attributes of rice through the application of CRISPR/Cas technology. The role of alleles generated by editing in gene regulation in a particular genetic pathway is crucial for editing to be effective. It is recommended that functional validation of the targeted allele be done across two or three diverse genetic backgrounds to determine its value. The role that an allele plays for a given trait is determined by (1) the type of trait, (2) the position of the gene in the molecular pathway, (3) the role of the gene in trait expression (phenotype), and (4) the genetic background in which it is expressed. While this is a customized tool, our understanding of gene networks and the information about superior alleles impedes advancement in this field. This perspective highlights the progress and obstacles in gene editing to achieve the desired plant phenotype. The editing of an allele may sometimes yield diverse phenotypic results depending on the plant genetic background, with certain cultivars achieving the desired phenotype albeit with pleiotropic effects, while others fail to elicit the intended phenotype at all. This challenge can be addressed by prioritizing the identification of the right candidate and specific motifs in the regulatory regions as potential targets rather than directly intervening in coding sequences.

## SUPPLEMENTARY DATA

Supplementary data are available at Annals of Botany online and consist of the following.

Table S1: list of 39 tomato genotypes assessed for SNP variation. Table S2: SNP analysis across 39 tomato genotypes for two fruit quality-related genes, *Solyc04g040190* (*CycB*) and *Solyc05g050010* (*ACS4*). This study identified three haplotypes in the coding region of *Solyc04g040190* and five haplotypes in *Solyc05g050010*. The position and type of variation observed for the two loci (acronyms 04g and 05g) are indicated for the 39 tomato genotypes (details of genotypes are given in [Supplementary Data Table S1](#); 1–32 are cultivated while W1–W8 are wild species). Table S3: SNP analysis for two yield-related rice genes, *OsSPL16* (*Os08g41940*) and *OsAAP3* (*Os11g09020*), across 3024 genotypes using the Rice SNP-seek Database. Three major haplotypes belonging to 12 subpopulations were identified in the coding and promoter regions of *Os08g41940*. Similarly, three and two haplotypes were found in the coding and 1-kb upstream region of *Os11g09020*, respectively. The haplotypes (hap) along with subpopulation classification, the number of accessions, the frequency of each haplotype and the position and type of variation are indicated.

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The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

Conceptualization: W.T.; writing: M.M.; writing – review and editing: M.M., M.R. and W.T.

## DATA AVAILABILITY

All data supporting the findings of this study are available within the paper and its supplementary materials published online.

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