REVIEW

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Rice Promoter Editing: An Efficient Genetic Improvement Strategy

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Abstract

Gene expression levels in rice (*Oryza sativa* L.) and other plant species are determined by the promoters, which directly control phenotypic characteristics. As essential components of genes, promoters regulate the intensity, location, and timing of gene expression. They contain numerous regulatory elements and serve as binding sites for proteins that modulate transcription, including transcription factors and RNA polymerases. Genome editing can alter promoter sequences, thereby precisely modifying the expression patterns of specific genes, and ultimately affecting the morphology, quality, and resistance of rice. This paper summarizes research on rice promoter editing conducted in recent years, focusing on improvements in yield, heading date, quality, and disease resistance. It is expected to inform the application of promoter editing and encourage further research and development in crop genetic improvement with promote.

Keywords Promoter editing, CRISPR/Cas, Rice improvement, Gene regulation

Introduction

The phenotype of rice is influenced by both genetic and environmental factors (Wu et al. 2020; Chen et al. 2024; Ahmad et al. 2024). Genetic modification offers the potential for stable phenotypic performance by altering its genetic information (Zeb et al. 2022; Ahmad et al. 2022a, b). Common strategies include introducing genes from exogenous species, modulating gene expression levels, and completely disrupting gene function (Luo et al. 2024; Zeng et al. 2020a, b). Consequently, a variety of gene editing techniques have been developed and employed to achieve these goals, such as TALENs (Becker and Boch 2021), ZFNs (Gupta and Musunuru 2014), CRISPR/Cas (Riaz et al. 2022; Shaheen et al. 2023), Prime Editing (Xu et al. 2020; Ahmad et al. 2022a, b), and Base Editing (Li et al. 2023a, b, c; Tabassum et al. 2021; Monsur et al. 2020). The advent of these technologies has revolutionized the precise manipulation of targeted nucleic acids, profoundly impacting modern rice breeding efforts (Lu et al. 2018; Ahmad et al. 2021a, b). For a long period in the past, rice gene editing had concentrated on modification of the coding regions of functional genes. While this approach has yielded some positive results, it has also revealed certain drawbacks. For example, leading to unwanted abnormal phenotypes such as cessation of flowering, reduced yield and increased susceptibility to diseases (Fan et al. 2014; Li et al. 2020; Tan et al. 2018; Zhao et al. 2015). Additionally, deletion of entire promoter may result in damage to the three-dimensional genome structure and cause severe phenotypic changes

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(Spielmann et al. 2018). Consequently, researchers have observed that the most effective approach to addressing these limitations may involve mild regulation of gene expression.

In recent years, molecular breeders have shifted their focus from coding sequences to other genomic regions, with particular emphasis on promoters (Jia et al. 2022; Liu et al. 2021a, b; Peng et al. 2017). Promoters are DNA sequences comprising of several thousand base pairs (bp) located upstream of the transcribed region of genes. They typically contain multiple transcriptional regulatory elements, known as cis-regulatory elements (CREs), which regulate gene expression by binding to specific proteins (Marand et al. 2023). The core promoter is typically defined as the region approximately ± 50 bp on either side of the transcription start site (TSS) (Bernardini et al. 2023), which plays a pivotal role in regulating the initiation and direction of transcription (Haberle and Stark 2018). The proximal promoter with many primary regulatory elements is found approximately 250 bp upstream from the TSS. It is the binding site for many transcription factors (Thrall et al. 2024). The distal promoter, situated further upstream from the proximal promoter, also contains lots of cis-regulatory elements (Hausman 2022). Furthermore, upstream of the coding sequence contains the 5′ untranslated region (5′UTR) and the upstream open reading frames (uORFs) located therein. The 5′UTR, which lies between TSS and the first coding base, plays a non-negligible role in transcription regulation (Chung et al. 2006; Samadder et al. 2017). uORFs, potential open reading frames within the 5′UTR, have been reported to regulate the transcription or translation of downstream main open reading frame (mORF) (Combier et al. 2008; Xu et al. 2017). The promoter region contains both known and potential factors that regulate gene expression, so it has received attention from researchers in recent years. Consequently, editing this region has been progressively studied in depth. It has been demonstrated that editing promoter sequences can be an effective means for mastering gene expression regulation (Li et al. 2017; Zeng et al. 2020a, b; Wang et al. 2024). Editing crop promoters had the potential to generate novel alleles that regulated the expression of target genes on a large range (Rodriguez-Leal et al. 2017). Mutations in coding sequences often lead to complete disruption of gene function (Wittkopp and Kalay 2012). At some point, it makes more sense to efficiently reduce gene expression without completely disrupting gene function, thereby achieving the desired results with no or only negligible negative effects. Mutations in CREs tends to alter the spatiotemporal expression patterns and intensity of genes (Wu et al. 2021a, b). Editing specific CREs in promoter region was considered a practical strategy for reducing transcript levels (Haberle and Stark 2018). In rice promoter editing, fine-tuning target gene expression through promoter editing had successfully minimized adverse effects on plant growth and development (Wang et al. 2021a, b, c). Numerous examples demonstrated that regulating gene expression by editing CREs in the promoter regions of target genes is an effective strategy for genetic improvement of rice (Li et al. 2023a, b, c) and aligns with the theories and practices of modern rice breeding.

As a globally essential food crop, rice serves as a staple for billions, particularly in Asian countries. The focus on enhancing rice traits, such as yield, quality, and resistance, has never been more critical. Although improving rice yield has traditionally been a primary focus of breeders, it is notable that only a few studies have directly addressed yield traits. Instead, the majority of researchers concentrated their efforts on improving rice heading date, eating and cooking qualities (ECQ) and bacterial disease resistance, with only a few studies focusing on other aspects. This trend is somewhat unexpected, but it is worth noting that most existing literatures also considered the impact of promoter region editing on yield when examining other traits. Therefore, promoter gene editing, a powerful tool in genetic improvement, holds vast potential for application in rice production and may serve as a model for breeding other crops.

Moreover, as a model organism among crops, rice offers several advantages for genetic research: it is one of the earliest plants to have its genome sequenced, it is diploid with a relatively small genome, and the costs associated with its well-established genetic transformation are low. These factors make rice uniquely suited for promoter editing studies, providing a fertile ground for the validation and optimization of this technology. Furthermore, rice can rapidly generate large amounts of genetic materials, facilitating the swift validation and refinement of promoter editing techniques. Then, the significance of rice promoter editing extends far beyond this crop; it holds promise for advancing the entire field of plant genetics. In addition, the wealth of wild rice germplasm and regionally cultivated rice varieties, particularly in Asia, adds another layer of complexity and opportunity. The regional specificity and strong local adaptation of commercial rice varieties necessitate tailored promoter editing strategies to accommodate the genetic diversity found in different varieties. This specificity is crucial for the successful improvement of rice varieties. Thus, a comprehensive review of the research progress in rice promoter editing is not only timely but essential.

This paper provides a comprehensive review of recent literature on rice promoter region editing (Table 1). As gene editing technology continues to advance with greater precision and efficiency, it is anticipated that an

Table 1 List of some published reports on rice promoter editing

Target gene	Short description	Editing system	Edited region	Effect on gene expression	Major phenotypes	References
RFT1	Florigen gene	CRISPR/Cas9	A 0.5 kb region in the promoter	Significantly decreased	Delayed heading date	Zhou et al. (2024a, b)
RMS	Cytoplasmic male sterility restoration gene	CRISPR/Cas9	The promoter region located between the CDS of PPR2 and RMS	Decreased	Partial restoration of fertility	Suketomo et al. (2020)
Hd1, Ghd7, DTH8	Hd1: Photoperiod-sensitivity gene; Ghd7, DTH8: Grain number per panicle, plant height and heading date genes	HMP based on CRISPR/Cas9	A 2.0 kb region in the promoter (randomly and evenly)	Decreased to varying degrees	A range of quantitative variation of heading date	Zhou et al. (2024a, b)
Hd2	Grain number per panicle, plant height and heading date gene	CRISPR/Cas9	Three uORFs in 5′ leader sequence	Transcription levels remained unchanged but protein levels increased	Delayed heading date	Liu et al. (2021a, b)
Wx1764178(A)	An allele of Wx (granule-bound starch synthase gene)	CRISPR/Cas9	Near the A-SNP in the promoter	Decreased	Reduced apparent amylose content	Tang et al. (2024)
Wxa	An allele of Wx	CRISPR/Cas9	A 500–760 bp region in the promoter	Decreased	Reduced amylose content; Improved chalkiness	
Wxb	An allele of Wx	CRISPR/Cas9	Near several CREs in the promoter; Near TATA-box	Slightly decreased	Various changes in amylose content	Huang et al. (2020)
Wxa	Described above	CRISPR/Cas9	Near several putative CREs in the promoter; Near endosperm-box, A-box and CAAT-box; 5′ UTR intronic splicing site (5′UISS)	Decreased	Reduced amylose content; Changed viscosity index	Zeng et al. (2020a, b)
NAS2	Nicotianamine synthase gene	CRISPR/Cas9	ARR1AT element in the promoter	Significantly increased	Increased Zn/Fe concentration; Improved panicle branching and seed development	Ludwig et al. (2024)
Ehd1	Early heading date gene	CRISPR/Cas9	A 3.0 kb region of the promoter (evenly distribution), one of the targets is located on an uORF	Decreased to varying degrees	Delayed heading date; Increased yield	Li et al. (2023a, b, c)
GWD1	α-glucan, water dikinase gene	CRISPR/Cas9	Near several CREs in the promoter	Decreased	Reduced chalkiness; Slightly altered grain shape	Wang et al. (2021a, b, c)
SLG7	Major QTL controlling grain length and width	CRISPR/Cas9	Near several CREs in the promoter, one is closed to a putative AC II element	Increased	Reduced chalkiness; Slightly Changed grain shape	Tan et al. (2023)

Table 1 (continued)

Target gene	Short description	Editing system	Edited region	Effect on gene expression	Major phenotypes	References
SWEET11, SWEET13, SWEET14	Bacterial blight susceptibility genes	CRISPR/Cas9	Several short EBEs in the promoters	/	Powerful and spectral resistance	Oliva et al. (2019)
SWEET11a, SWEET13, SWEET14	Bacterial blight susceptibility genes	CRISPR/Cas9	Near the EBEs in the promoters	Insensitive to induction of expression by specific TALEs	Complex BLB resistance or susceptibility; Affected pollen viability seed setting rate	Liu et al. (2024)
SWEET11, SWEET13, SWEET14, SWEET15	Bacterial blight susceptibility genes	CRISPR/Cas9	Near the EBEs in the promoters	/	Endowed BLB resistance	Eom et al. (2019)
xa23	An inactive allele of *Xa23* (A bacterial blight resistance gene)	Combining CRISPR/SpCas9 with dsODNs	The promoter of *xa23*	Sensitive to induction of expression by specific TALEs	Acquired BLB resistance to homologous EBEs	Kumar et al. (2023)
SWEET11, SWEET14, SULTR3;6	*SWEET11, SWEET14*: Described above; *OsSULTR3;6*: Bacterial Leaf Streak susceptibility gene	CRISPR/Cas9	EBEs in *SWEET11, SWEET14 and SULTR3;6*	Insensitive to induction of expression by specific TALEs	Acquired BLB resistance and BLS resistance respectively	Ni et al. (2021)
SWEET14	Bacterial blight susceptibility gene	CRISPR/Cas9	EBE$_{AvrXa7,PthXo3,TalF}$ in the promoter	Insensitive to induction of expression by specific TALEs	Endowed BLB resistance	Duy et al. (2021)
xa23	Described above	Prime editing	The promoter of *xa23*	Sensitive to induction of expression by specific TALEs	Restored resistance	Gupta et al. (2023)
TCP4^{M-}	TCP family transcription factor	/	A miniature inverted-repeat transposable element (MITE)	Significantly decreased	Increased tiller number; Shortened seed length	Zhang et al. (2024)
GBSS1 (Wx), GS3, D2, D11, SD1, D18	*GBSS1*: granule-bound starch synthase gene; *GS3*: major QTL for grain length and weight; *OsD2*: dwarf gene; *OsD11*: dwarf, grain number and size gene, *OsSD1*: gibberellin 20-oxidase gene; *OsD18*: dwarf gene	CRISPR/Cas12a promoter editing (CAPE) system	Five key regions in the promoters, respectively	Continuous changes	Quantitative trait variation of starch content, grain size, thousand-grain weight, height, panicle length, seeds per panicle, respectively; Semi-dwarfsm; anti-lodging	Zhou et al. (2023)
xa13 (SWEET11)	Bacterial blight susceptibility gene	CRISPR/Cas9	Pathogen-induced element in the promoter	Insensitive to induction of expression by specific TALEs	Endowed BLB resistance	Li et al. (2022)
V-Ppase	Vacuolar H$^+$ Translocating Pyrophosphatase (V-PPase) gene	CRISPR/Cas9	GATA promoter element	Significantly decreased	Reduced chalkiness; Slightly altered grain shape	Gann et al. (2022)

Table 1 (continued)

Target gene	Short description	Editing system	Edited region	Effect on gene expression	Major phenotypes	References
V-Ppase	Vacuolar H+ Translocating Pyrophosphatase (V-PPase) gene	CRISPR/Cas9	GATA promoter element	Significantly decreased	Reduced germination and seedling growth rate; Decreased sucrose content	Dharwadker et al. (2022)
xa23	An inactive allele of Xa23	CRISPR/Cas9-mediated precise homology directed repair(HdR)	The promoter of xa23	Sensitive to induction of expression by specific TALEs	Restored resistance	Wei et al. (2021)
SWEET11a, SWEET13, SWEET14	Bacterial blight susceptibility genes	Hybrid Cas9/Cpf1 editing system	EBE_PthXo1/PthXo1A/PthXo2A in the promoters of SWEET11a and SWEET13, EBE_AvrXa7,PthXo3,TalF in the promoter of SWEET14	/	Gained broad spectrum BLB resistance	Schepler-Luu et al. (2023)
xa13(SWEET11)	Bacterial blight susceptibility genes	CRISPR/Cas12a	Key bacterial protein-binding site in the UPT box of Xa13 promoter	Insensitive to induction of expression by specific TALEs	Improved bacterial blight resistance	Yu et al. (2021)
xa13(SWEET11)	Bacterial blight susceptibility gene	CRISPR/Cas9	A bacterial blight-inducible element in the promoter	Insensitive to induction of expression by specific TALEs	Enhanced BLB resistance	Li et al. (2022)
SULTR3;6	Bacterial leaf streak (BLS) susceptibility genes	CRISPR/Cas9	Near EBE_Tal2g/Tal5d in the promoter	Insensitive to induction of expression by specific TALEs	Gained BLS resistance	Xu et al. (2021)
SWEET14	Bacterial blight susceptibility gene	CRISPR/Cas9	Four EBEs in the promoter	Insensitive to induction of expression by specific TALEs	Gained BLB resistance to respective TALEs	Zafar et al. (2020)
OsF3H^03g, OsF3H^04g	Bacterial leaf streak (BLS) susceptibility genes	CRISPR/Cas9	Effector-binding element in the promoters	Insensitive to induction of expression by specific TALEs	Specifically enhance resistance to Tal2b- or Tal2c-transferring strains	Wu et al. (2021a, b)
OsSWEET11a	Bacterial blight susceptibility genes	CRISPR/Cas9	EBE_Tal6b/AvrXa27A in the promoter	/	Gained Tal6b/AvrXa27A-dependent susceptibility	Xu et al. (2024)
IPA1	Ideal plant architecture	CRISPR/Cas9	Cis-regulatory regions (CRRs) in the promoter; 5′UTR	Altered in tissue-specific manners;	Increase tiller number and panicle size simultaneously	Song et al. (2022)
Wx	Granule-bound starch synthase gene	CRISPR/FrCas9	Predicted atypical TATA box sequence in the core promoter	Significantly decreased	Lower transcript abundance of Wx and reduced amylose content	Wang et al. (2024)

The "/" symbol indicates that it was not mentioned in the paper

increasing number of studies will focus on promoter region editing in rice and other crops. We believe that this comprehensive review will serve as a valuable reference for future research on promoter editing in other plant species, and will help drive further advancements in crop genetic improvement and agricultural sustainability.

Research Progress on Promoter Region Editing of Rice

In recent years, studies on editing of the promoter region in rice have focused on regulation of heading date, improvement of eating and cooking quality, and enhancement of bacterial disease resistance. In addition, a few studies have explored the improvement of rice yield, fertility restoration of male sterile lines, and enhancement of micronutrient content.

Regulation of Yield-Related Traits

The breeding of high-yielding rice is a topic that is consistently addressed in the field of rice cultivation. Despite the successes of existing high-yielding varieties, breeders continually strive for higher and more consistent yields with unremitting efforts. Rice yield is primarily determined by tillering and panicle architecture traits (Wen et al. 2024), including panicle number, grain number per panicle, and grain weight (Amin et al 2024; Wang and Li 2005). These traits exhibit complex quantitative variation among different rice varieties, as they are typical quantitative traits. To generate a wide range of Quantitative Trait Variants (QTVs) in rice, a CRISPR/Cas12a promoter editing (CAPE) system was developed, which was based on a promoter Key-Region (KR) estimating model and an efficient CRISPR/Cas12a-based multiplexed or singular editing system (Zhou et al. 2023). Experimental results with promoter editing of multiple rice genes demonstrated the efficacy of this system in creating QTVs. It was observed that editing KRs with high total scores produced more obvious phenotypes. For instance, promoter-edited *OsGBSS1* (*Wx*) and *OsGS3* lines demonstrated continuous variation in starch content and seed size (Zhou et al. 2023). Furthermore, the expression level of *OsGBSS1* correlated strongly with amylose content (AC) (Zhou et al. 2023). Additionally, QTVs for seed length and width were also observed (Zhou et al. 2023). A comparative analysis of two representative promoter-edited lines revealed no differences in morphology, height, or main panicle length compared to wild-type (WT) (Zhou et al. 2023). Promoter-edited *OsD2* lines showed a gradual reduction in *OsD2* expression, which was accompanied by a decrease in plant height. Nevertheless, main panicle length, seed number, seed size, and 1,000-grain weight were affected to varying extents (Zhou et al. 2023). Concurrently, promoter editing of *OsD11* yielded

outcomes comparable to those of *OsD2* (Zhou et al. 2023). *OsSD1* (also known as *GA20ox*) has been successfully employed to generate rice varieties exhibiting semi-dwarfing characteristics while maintaining yield stability (Qiao and Zhao 2011). Two promoter-edited *OsSD1* lines also exhibited dwarfing traits and showed no differences in grain size or 1000-grain weight compared to WT and null-mutant controls. The promoter-edited *OsD18* lines demonstrated a continuous range of variation in plant height, from 64 to 98 cm, with reduced *OsD18* expression (Zhou et al. 2023). Additionally, main panicle length and grain number exhibited intermediate phenotypes between *OsD18* knockout mutants and the WT (Zhou et al. 2023). In the field, wild-type plants exhibited a pronounced lodging phenotype, whereas *OsD18* promoter-edited lines demonstrated resistance to lodging (Zhou et al. 2023). This study highlighted that editing the *OsD18* promoter can produce semi-dwarfing QTVs without a significant loss of yield, which is referred to as a "quantitative green revolution trait". The most notable aspect of this research is the development of the robust CAPE system, which effectively introduces QTVs related to grain starch content, grain size, and plant height. This study not only represents a technological innovation in promoter editing but also validates the potential of promoter editing to introduce QTVs in rice. The refinement and widespread adoption of the CAPE system are expected to greatly advance promoter editing research, facilitate the creation of diverse trait variations, and ultimately contribute to the improvement of rice and other crops.

Rice breeding is often constrained by trade-offs among different agronomic traits, including the penalties of yield by plant immunity, dilution effect of plant nutrition, and negative correlations among plant architecture components (Nelson et al. 2018; Takatsuji 2017; Wang et al. 2021a, b, c; Wang et al. 2021a, b, c). These trade-offs, particularly among traits like tiller number, grain number per panicle and grain weight, limit overall rice yield. Many of these trade-offs can be attributed to genetic pleiotropy (Song et al. 2022). Pleiotropic genes affecting multiple traits may result in undesirable consequences. When attempting to regulate a specific trait by modifying a pleiotropic gene, there is a possibility that the modification may have an adverse effect on other traits associated with that gene. Successfully breaking the pleiotropy allows for the full utilization of beneficial effects while overcoming its adverse effects on other traits. In a previous study, researchers demonstrated that editing the promoter region of *IPA1* effectively reduced its pleiotropy (Song et al. 2022). They discovered that certain promoter fragments deletion mutants can influence *IPA1* expression in a tissue-specific manner, thereby reducing *IPA1* pleiotropy and producing extensive phenotypic variation

in tiller number and panicle size (Song et al. 2022). One such promoter-edited lines, IPA1-Pro10,which contained a 54 bp deletion, exhibited an increase in both tiller number and panicle size, leading to a greatly improvement in rice yield by overcoming their trade-offs (Song et al. 2022). Field experiments showed that the IPA1-Pro10 exhibited a 7.2% increase in tiller number per plant and an 8.5% increase in grain number per panicle compared to ZH11 (Song et al. 2022). Additionally, IPA1-Pro10 demonstrated a 15.9% increase in yield while maintaining a similar heading date (Song et al. 2022). To elucidate the mechanism underlying this synergistic increase in yield-related traits, researchers conducted further investigations and discovered that the transcription factor An-1 (Luo et al. 2013) could directly bind to the GCGCGTGT motif on the promoter of *IPA1*, repressing the expression of *IPA1* in the spikelet (Song et al. 2022). This suggested that An-1 specifically restrains spikelet and secondary meristem of the panicle. The deletion of this region resulted in altered tissue-specific expression of *IPA1* and separated the pleiotropy of both *IPA1* and An-1 to overcome agronomic trade-offs (Song et al. 2022). This study demonstrates that a tiling-deletion screen for cis-regulatory regions (CRRs) holds promise for reducing gene pleiotropy caused by different spatial–temporal functions of the same gene, which is expected to address the breeding difficulty resulting from trade-offs. As many genes affect rice yield (Dong et al. 2022), future research should delve deeper into these genes, especially using promoter editing technology.

Regulation of Heading Date

Flowering date is an important trait for rice adaptation to different light conditions in multiple places. Fine-tuning the heading date of desirable rice varieties has received much attention from breeders. There are many genes that can control the heading data of rice (Chen et al. 2022). Typically, delaying the heading date in rice is achieved through two main approaches: either elevation of the expression of floral inhibitors, or reduction of the expression of floral inducers (Liu et al. 2021a, b). Direct editing of the coding region of flowering genes often resulted in null mutations, which can significantly delay or even prevent the heading of rice (Zhao et al. 2015). Consequently, mutations caused by editing coding regions often have no practical application value. A more practical goal for rice breeders is to create a series of rice lines with different heading dates by fine-tuning the expression of flowering genes. *Rice Flowering Locus T1* (*RFT1*) was a significant floral regulator under long-day (LD) conditions (Tsuji et al. 2011). *RFT1* formed a ternary complex (Florigen activation complex, FAC) with 14-3-3 protein and the transcription factor OsFD1, which promoted flowering

under LD conditions (Peng et al. 2021). Recently, a study successfully created mutants with multiple promoter sequence variations by editing a 0.5 kb region in the promoter of *RFT1* (Zhou et al. 2024a, b). These mutants exhibited delayed heading date ranging from 1.4 to 9.2 days, accompanied by reduced expression of flower-forming genes *RFT1* and *Heading date 3a* (*Hd3a*) (Zhou et al. 2024a, b). The researchers also assessed the promoter activity of the *RFT1* promoter mutant, which showed reduced promoter activity (Zhou et al. 2024a, b). In addition, their investigations showed that other agronomic traits, such as plant height, panicle number, and 1,000-grain weight, in the mutants were not significantly different from the WT (Zhou et al. 2024a, b).

Heading date 1 (*Hd1*), *Heading date 7* (*Ghd7*), and *Days to heading 8* (*DTH8*) were three genes that act mainly upstream of *Early heading date 1* (*Ehd1*), and their combinations largely determined the photoperiod sensitivity and geographic environment adaptation of rice (Zhang et al. 2015a, b). *Hd1* was a photoperiod-sensitive gene that affected heading date and maturity in rice, and both *Ghd7* and *DTH8* were important flowering suppressors (Xue et al. 2008; Wei et al. 2010). Using the High-Efficiency Multiplex Promoter-Targeting (HMP) system, the 2.0 kb promoter regions of *Hd1*, *Ghd7*, and *DTH8* were randomly and uniformly edited, and new alleles in their cis-regulatory regions were generated (Zhou et al. 2024a, b). These mutants exhibited a range of heading dates, for example, the promoter-edited mutants of *Hd1* had heading dates ranging from about 73 days like the *Hd1* null mutation to about 107 days like the WT. Similar phenotypes were observed in mutant lines of *Ghd7* and *DTH8* (Zhou et al. 2024a, b). Notably, several of these mutant lines exhibited precocious heading phenotypes close to the *Hd1* null mutation (Zhou et al. 2024a, b), suggesting that the promoter mutations in these lines are almost equivalent to the null mutation.

The early heading gene *Ehd1* promoted rice flowering by activating the expression of flowering genes *Hd3a* and *RFT1* (Doi et al. 2004), while *Hd2* was a repressor of *Ehd1* (Hori et al. 2016). In previous study, the heading date of rice was extended by surging the expression of flowering repressors (Liu et al. 2021a, b). Then, three uORFs in the *Hd2* leader sequence were identified and edited, and mutant lines with 4–11 flowering days later than the WT were successfully obtained (Liu et al. 2021a, b). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) results showed a significant decrease in the expression of *Ehd1*, *Hd3a* and *RFT1* (Liu et al. 2021a, b). Interestingly, the transcript level of *Hd2* remained unchanged, but a follow-up study showed that the protein level of *Hd2* was actually increased (Liu et al. 2021a, b), suggesting that the uORF of *Hd2* can inhibit

the translation without affecting transcription. This study provides new insights into target selection for promoter editing.

Ehd1 not only regulated rice heading date but also affected yield-related agronomic traits (Cho et al. 2016; Doi et al. 2004). Several *Ehd1* promoter mutants with editing the nearby regions of some certain CREs in *Ehd1* promoter exhibited varying degrees of reduced expression levels of *Ehd1*, *Hd3a* and *RFT1* (Li et al. 2023a, b, c). This was different from the situation where the *Ehd1* null mutation with editing of coding region of *Ehd1* led to almost completely suppressed expression of *Hd3a* and *RFT1* (Li et al. 2023a, b, c). Furthermore, promoter mutants displayed delayed heading dates and enhanced some yield-related traits (Li et al. 2023a, b, c). The uORF mutants exhibited similar phenotypes (Li et al. 2023a, b, c), indicating that this uORF positively regulates the function of *Ehd1*.

Improvement of Quality

As living standards rise, people are paying greater attention to the flavor and appearance of rice quality. Studies have demonstrated that gene editing certain genes can improve quality of rice (Hui et al. 2022; Mao et al. 2021). Amylose content (AC) is a significant indicator of the eating and cooking quality (ECQ) of rice (Li et al. 2016). In general, rice grains with higher AC and lower gel consistency showed poorer taste profile, whereas grains with moderate AC (15–20%) and higher gel consistency (60–80 mm) exhibited superior taste profile for the majority of population (Zeng et al. 2020a, b). *Wx* (*OsGBSS1*) was regarded as the most pivotal gene regulating AC (Sano 1984; Zhu et al. 2024). The *Wx*-encoded granule-bound starch synthase 1 (GBSS1) was the primary enzyme responsible for influencing AC (Mikami et al. 2008; Zhang et al. 2019). Natural variations in *Wx* gave rise to different levels of AC, as well as variations in ECQ among different rice varieties (Zhang et al. 2019). In addition to regulating AC and ECQ, *Wx* also affected gel consistency, water absorption, and elongation of cooked rice (Kim et al. 2021; Tian et al. 2009; Zhang et al. 2021). This evidence demonstrates that *Wx* plays a significant role in determining the ECQ of rice. Based on the regulatory role of *Wx* on AC, a lot of researchers believe that editing *Wx* to regulate AC for improving rice quality is a viable strategy.

Moreover, it has been demonstrated that moderate regulation of AC could be achieved by precisely regulating the expression of *Wx* (Huang et al. 2020). *Wx1764178A* identified by Tang et al. (2024) was an allele of *Wx* that had an adenine variant SNP (A-SNP) in its promoter region. In previous study, the CRISPR/Cas9 system was employed to target the sequence in the vicinity of the A-SNP in the promoter of this allele for gene editing (Huang et al. 2020). Compared to the WT, the promoter-edited lines exhibited reduced *Wx* expression, with a reduction in apparent amylose content (AAC) of approximately 10% (Huang et al. 2020). It is noteworthy that the target is situated 2,500 bp away from the upstream region of the *Wx* promoter. This suggests that the discovery and utilization of polymorphic sites beyond 2000 bp upstream of the promoter may also have potential for applications.

Reducing rice chalkiness is also a crucial aspect in achieving high-quality rice. *Wxa*, which was also an allele of *Wx*, contributed to the production of rice with a high AC (25–30%), which negatively affected the flavor of rice (Wang et al. 1995). By modifying the region between 500 and 760 base pairs upstream of the *Wxa* promoter, edited lines demonstrated a notable decrease in AC (Li et al. 2023a, b, c). Furthermore, these edited lines were accompanied by an improvement in chalky grain percentage (CGP) and chalk grain grade (CGG), without any adverse effects on other agronomic traits (Li et al. 2023a, b, c). Other studies have also conducted research on promoter editing in *Wxa*. Gene editing of several CREs, including Endosperm-box, A-box, CAAT-box and 5'UTR intronic splicing site (5'UISS), in the *Wxa* promoter region has been conducted (Zeng et al. 2020a, b). The findings indicated a notable decline in *Wxa* mRNA levels among a portion of promoter region element mutants, as well as across the entirety of 5′UISS mutants. Rapid visco analysis of starch quality indicated that the viscosity index of the edited lines correlated with their ACs, exhibiting various degrees of variability (Zeng et al. 2020a, b). Meantime, the primary agronomic characteristics of the mutants, including grain weight, grain length, grain width, and plant height, were not significantly different from those of the WT (Zeng et al. 2020a, b).

Wxb was a weak allele of *Wx* that produced moderate AC (15–18%) (Isshiki et al. 1998). By editing the region near the TATA-box of the *Wxb* promoter, mutant lines were successfully generated with reduced the expression level of *Wx* (Huang et al. 2020). Some promoter-edited lines exhibited markedly diminished AC, whereas others demonstrated no discernible alterations (Huang et al. 2020). Notably, one edited line demonstrated a moderate reduction in AC under a variety of growth conditions (Huang et al. 2020). This particular line was distinguished by the alteration of only the bases situated in the vicinity of the TATA box, without any impact on other sequences, resulting in a significant change in *Wx* expression (Huang et al. 2020). This study demonstrates that targeting the core region of a gene promoter for editing is a viable method for regulating gene expression.

A recent study introduced an engineered CRISPR/FrCas9 system from Faecalibaculum rodentium for efficient plant genome editing, overcoming challenges with TA-rich core promoters (Wang et al. 2024). The study showed that the system can efficiently edit the core promoter region of the *Wx* gene. The transcript abundance of *Wx* in the promoter edited lines were reduced to a certain extent, accompanied by a decrease in AC (Wang et al. 2024). This research highlights the high efficiency of FrCas9-mediated core promoter editing in rice, and its potential for versatile base editing as well as precise gene expression regulation.

In addition to *Wx*, *Glucan*, *Water-Dikinase 1* (*GWD1*) was a pivotal gene that regulated both the degradation and synthesis of rice starch (Ritte et al. 2002). GWD1 catalyzed the reaction of α-glucan and adenosine triphosphate (ATP) with water to produce α-glucan phosphate monoesters, adenosine monophosphate (AMP), and orthophosphate (Ritte et al. 2002). Additionally, GWD1 catalyzed the phosphorylation of starch, regulating both starch degradation and synthesis (Mikkelsen et al. 2005; Skeffington et al. 2019; Xu et al. 2017). Using the CRISPR/Cas9 system, researchers successfully edited the promoter of *GWD1*, generating *gwd1* weak mutants (Wang et al. 2021a, b, c). These promoter mutant lines exhibited a notable reduction in chalky grain grade and chalky grain percentage, although there was only a slight alteration in grain shape (Wang et al. 2021a, b, c). Compared to WT, the transcript levels of *GWD1* in the promoter-edited mutants were approximately 50% of those in WT. Additionally, the peak viscosity (PKV), hot paste viscosity (HPV), cool paste viscosity (CPV), and breakdown viscosity (BDV) of the *gwd1* mutant rice flour exhibited a slight decrease, whereas the setback viscosity (SBV) demonstrated an increase compared with WT (Wang et al. 2021a, b, c). In contrast to the significant reduction in grain yield observed in the null mutant of *GWD1* (Hirose et al. 2013), the authors noted that the mutation caused by promoter editing of *GWD1* had relatively mild effects and did not significantly impact yield-related traits (Wang et al. 2021a, b, c).This implied that the ECQ of the promoter-edited lines was somewhat decreased (Wang et al. 2021a, b, c). In contrast to the significant reduction in grain yield observed in the null mutant of *GWD1* (Hirose et al. 2013), the authors indicated that the mutation caused by promoter editing of *GWD1* had relatively mild effects and did not affect yield-related traits (Wang et al. 2021a, b, c).

SLG7, an allele of *GL7* and *GW7*, was associated with grain shape and starch quality (Wang et al. 2015). AH2 was a MYB-domain protein with a crucial target site AC II element (ACCAATCC) that played a crucial role in the development of grain and hull (Ren et al. 2019). Research has shown that AH2 was essential for determining the development of hull epidermis, palea identity, and grain size (Ren et al. 2019). Promoter-edited lines of *SLG7* were created by targeting several CREs on the *SLG7* promoter (Tan et al. 2023). The researchers identified the AC II element at one of the mutation sites, suggesting that mutation at this site may affect the interaction between AH2 and *SLG7* and thus affecting *SLG7* expression (Tan et al. 2023). Some *SLG7* promoter-edited mutants exhibited elevated *SLG7* expression, reduced chalkiness, longer grains, higher amylose content, and decreased gel consistency, however, the yield per plant did not differ from that of WT (Tan et al. 2023). Further analysis revealed that the binding ability of AH2 to the mutant of *SLG7* promoter was significantly reduced, and the repressive effect of AH2 protein on the mutant promoter was also significantly reduced (Tan et al. 2023). This study highlights the significant potential of targeting CREs (e.g., transcription factor binding sites) in promoter regions to create beneficial mutations and improve rice traits.

Enhancement of Resistance to Bacterial Diseases

Rice is susceptible to a range of pathogenic agents, including fungi, bacteria, viruses, and other microorganisms. The most prevalent diseases such as sheath blight, blast, bacterial leaf blight, and tungro cause severe yield losses every year. In order to overcome these rice diseases, gene editing technology provides breeders with an effective way (Ahmad et al. 2020). Plant pathogens were able to identify specific signals from the host plant, which then initiated the infection process (Ontoy and Ham 2024). Bacteria employed specialized secretion systems to deliver effector molecules into plant cells, thereby modulating host responses and facilitating invasion (Zhang et al. 2022). Rice possesses a multitude of genes that confer resistance to specific pathogens, known as resistance genes (*R* genes). Conversely, there are susceptibility genes (*S* genes) that, when recognized by pathogens, make rice more vulnerable to disease. Two destructive rice bacterial diseases, bacterial leaf blight (BLB) and bacterial leaf streak (BLS), are caused by two different strains of the rice *Xanthomonas oryzae*: pv. *oryzae* (*Xoo*) and pv. *oryzicola* (*Xoc*) respectively. These pathogens are widely distributed and have significant negative impacts on global rice production. BLB was the most destructive disease, accounting for 40% of rice pest and disease losses (Shaheen et al. 2019; Syed-Ab-Rahman et al. 2020). *Xoo* and *Xoc* are two common pathogenic bacteria that promote rice disease by injecting Transcription-Activator-Like Effectors (TALEs) into rice plant cells. These TALEs recognized Effector-Binding Elements (EBEs) in the promoters of homologous host genes, which resulted in the activation of rice *S* genes (Bezrutczyk et al. 2018;

White et al. 2009). Consequently, many researchers have endeavored to regulate crucial *R* genes or *S* genes to develop rice varieties with the intention of obtaining disease resistance. This ultimately aims to breed rice varieties with enhanced resistance to the disease.

Moreover, it indicated that breeding resistant rice lines was the most effective way to tackle BLB caused by *Xoo* (Eom et al. 2019). The *Sugars Will Eventually Be Exported Transporter* (*SWEET*) gene family encodes sugar-transporting proteins required for normal plant growth and development. However, certain SWEET genes may be co-opted by pathogenic microorganisms to fulfill their nutrient requirements, making rice susceptible to disease (Gupta 2020). In rice, *OsSWEET11*, *OsSWEET13* and *OsSWEET14* have been identified as key susceptibility genes for BLB (Chen et al. 2010; Yuan and Wang 2013). The CRISPR/Cas9 system was employed to edit six EBEs in the promoters of *OsSWEET11*, *OsSWEET13*, and *OsSWEET14* (Oliva et al. 2019). This approach conferred robust broad-spectrum resistance to the edited lines without compromising agronomic traits in rice (Oliva et al. 2019). Evaluation of plant height, panicle length, tiller number, and fertility demonstrated that the majority of promoter-edited lines exhibited comparable performance to that of the WT parents (Oliva et al. 2019).

In a comparable study, genetically editing the EBEs in the promoters of *OsSWEET11a* (an allele of *OsSWEET11*), *OsSWEET13*, and *OsSWEET14* resulted in the production of various mutant lines that exhibited complex resistance or susceptibility to diverse bacterial strains (Liu et al. 2024). They also found that the deletion of EBE sites from the *OsSWEET11a* and *OsSWEET14* promoters resulted in a reduction in pollen viability and seed setting rate (Liu et al. 2024). Furthermore, EBE editing in the *OsSWEET13* promoter showed a positive effect on rice yield (Liu et al. 2024). Although EBEs editing may still have a negative impact on rice growth and development, it is reasonable to conclude that promoter editing is likely to have a less severe impact compared to null mutations that directly target the coding sequence.

By utilizing CRISPR/Cas9 to target EBEs in four of the SWEET genes (*OsSWEET11*, *OsSWEET13*, *OsSWEET14*, and *OsSWEET15*), promoter-edited lines were created to make changes in single or multiple EBEs (Eom et al. 2019). AXO1947, an African *Xoo* strain that causes bacterial blight in rice, exhibited a reduction in virulence in *OsSWEET14* single-knockout lines, yet was unable to infect the *SWEET13; SWEET14* double mutants (Eom et al. 2019). Growth and yield evaluations under greenhouse conditions revealed no discernible differences in performance for the *OsSWEET13* and *OsSWEET14* knockout mutant lines, when compared to the WT (Eom et al. 2019). Similarly, field experiments indicated that these lines exhibited comparable outcomes in terms of both yield and growth to the WT (Eom et al. 2019). The researchers concluded that EBE-edited lines in which the normal promoter function of *OsSWEET13* and *OsSWEET14* were affected would not cause yield loss based on this evidence (Eom et al. 2019). Although the experiments yielded positive results, they also indicated that the resistance conferred by the *SWEET* promoter editing variant that prevents binding of the TALEs would be rendered ineffective by the emergence of new pathogenic *Xoo* strains (Eom et al. 2019). Consequently, the *SWEET* promoter-edited lines may need to be used in combination with other *R* genes.

TALE Tal2g was a primary virulence factor of *Xoc*, which induced the expression of the *S* gene *OsSULTR3;6* in rice, thereby making it susceptible to disease (Cernadas et al. 2014). EBEs in the promoter regions of *Xoo* susceptibility genes (*OsSWEET11* and *OsSWEET14*), and *Xoc* susceptibility gene (*OsSULTR3;6*) were edited to obtain two promoter-edited rice lines of GT0105 and ZT0918 (Ni et al. 2021). These lines demonstrated significantly improved resistance to both *Xoo* and *Xoc* (Ni et al. 2021). Moreover, morphometric and other agronomic traits of GT0105 and ZT0918 exhibited no notable divergence from those of WT (Ni et al. 2021). This indicates that editing the EBEs in the promoters of *S* genes did not result in any alteration in the original biological functions of these genes. The result of qRT-PCR confirmed that the expression of *S* genes mentioned above in the edited rice lines was no longer responsive to pathogens carrying the corresponding TALEs (Ni et al. 2021). According to the study, this is the first instance in which engineered rice lines resistant to *Xoo* and *Xoc* had been produced through promoter editing of *S* genes. Furthermore, the editing process had not affected other agronomic traits. In a similar study, mutants edited at a 20-bp nucleotide target site overlapping the PthXo3, AvrXa7 and TalF EBEs were obtained in the *OsSWEET14* promoter (Duy et al. 2021). It is pertinent to note that the frequency of individuals with CRISPR/Cas9-induced mutations in T0 transgenic rice was 90% in this study (Duy et al. 2021). This high frequency of mutations serves to demonstrate the effectiveness of the CRISPR/Cas9 system as a tool for gene editing in rice. What's more, transgene-free plants with superiority of CRISPR/Cas9 technology can be generated from the majority of T1 segregation populations, without the necessity for laborious crossing or backcrossing procedures.

In addition, researchers have turned their attention to *R* genes, attempting to obtain enhanced resistance by editing the promoter regions of *R* genes. Two principal molecular strategies have emerged for improving rice resistance. The first strategy involves reducing the

susceptibility of *S* genes to TALEs. The second strategy focuses on insertion of EBEs induced by the causal bacterium into the dysfunctional promoters of *R* genes. Typically, the transcription of *R* genes was strictly controlled, but it can be activated by TALEs following pathogen attachment (Zhang et al. 2015a, b). This characteristic suggests that inserting EBEs into the promoters of less responsive *R* gene alleles could facilitate their recognition by TALEs, thus enhancing resistance. The *R* gene *Xa23* conferred the widest range of resistance to BLB in rice (Wang et al. 2014). The resistance of *Xa23* is a consequence of the interaction between *Xa23* and the TALE avrXa23 of *Xoo*. Upon infection of rice by *Xoo*, the secreted AvrXa23 can be captured by a 28-bp EBE (EBE$_{AvrXa23}$) in the promoter of *Xa23*, resulting in the activation of *Xa23* expression and the development of strong BLB resistance in rice. Unfortunately, EBE$_{AvrXa23}$ was present in only a few rice varieties, whereas *xa23s*, the alleles of *Xa23* that do not contain EBE$_{AvrXa23}$, were present in most sensitive rice genotypes (Cui et al. 2017). This characterization provides an opportunity for molecular breeders to improve BLB resistance in susceptible rice.

The insertion of EBE$_{AvrXa23}$ into the promoter region of the *xa23* allele in Nipponbare was achieved through CRISPR/Cas9-mediated precise homology directed repair (HdR) (Wei et al. 2021). This resulted in the transformation of BLB-susceptible rice variety with Nipponbare background into BLB-resistant line without significant effects on nutritional and yield traits (Wei et al. 2021). The investigation is groundbreaking, which represents a significant advancement in utilization of the highly efficacious BLB resistance gene *Xa23* and the application of genome editing technology in the genetic improvement of rice. It also serves as a paradigm for the study and utilization of pivotal genomic elements in rice. Later studies have confirmed the feasibility of this strategy. An efficient nonhomologous end-joining-mediated targeted insertion method was used to edit the promoter of *xa23* (Kumar et al. 2023). In this study, the insertion of EBE$_{AvrXa7/PthXo3}$ and EBE$_{TalC}$ into the *xa23* promoter region resulted in the activation of *xa23* expression, enabling it to respond to *Xoo* strains expressing the homologous TALEs and confer resistance to specific strains (Kumar et al. 2023). In another study, a modified prime editing (PE) system was used to knock-in EBE$_{AvrXa23}$ into the *xa23* promoter and conferred robust BLB resistance in susceptible rice (Gupta et al. 2023). This is the first report of using PE system to edit a non-functional rice *R* gene and efficiently incorporate a 30 bp cis-regulatory element. As the PE system continues to be refined and popularized, it is expected to provide a valuable tool for the efficient and precise editing of various genes, particularly in the promoter regions.

Other Improvements in Rice

Cytoplasmic male sterility (CMS) is a significant agricultural trait that is characterized by the absence of functional pollen due to abnormal gene expression in mitochondrion. The presence of nuclear-encoded fertility restorer (Rf) gene was able to restore the function of the pollen in male sterile lines (Kazama et al. 2016). *Retrograde-Regulated Male Sterility* (*RMS*) serves as a restorer gene for cytoplasmic male sterile lines of rice. RNA-interference-mediated silencing of *RMS* had been shown to restore fertility in CMS lines, however, over-expression of *RMS* in fertility-restoring lines had been observed to induce pollen abortion (Fujii and Toriyama 2009). Wild-type rice that normally expresses *RMS* remained completely sterile (Fujii and Toriyama 2009). The CRISPR-edited line created by targeting the *RMS* promoter between the coding sequence of *PPR2* (a Rf gene) and *RMS* was successfully developed, leading to partial restoration of fertility (Suketomo et al. 2020). The seed setting rate recovered to 15.9% in the T0 generation and 12.5% in the T1 generation, accompanied by a decrease in *RMS* expression (Suketomo et al. 2020).

Zinc (Zn) is a key micronutrient that plays a role in a multitude of biochemical processes in plants, such as serving as a co-factor for enzymes, participating in the synthesis of nucleotides and some plant hormones. Iron (Fe) is another essential micronutrient crucial for plant growth and development. It is the building block of several enzymes and plays a significant role in maintaining the structure of chloroplast. The *OsNAS2*, which encoded nicotinamide synthase in rice, had been demonstrated to enhance bioavailable Fe content with elevated expression level (Lee et al. 2017). *OsNAS2* showed great potential for Fe/Zn biofortification of rice endosperm (Johnson et al. 2011), and was regulated through a B-type OsRR-mediated signaling pathway (Gao et al. 2019). Disruption of the binding site of OsRR with the ARR1AT motif may diminish the inhibitory impact of the OsRR on *OsNAS2*, resulting in elevated intragrain Zn/Fe concentrations (Gao et al. 2019). CRISPR/Cas9 system was employed to edit the ARR1AT motif in the *OsNAS2* promoter, resulting in increased Zn/Fe concentrations in the promoter-edited lines (Ludwig et al. 2024). The 4 bp deletion-edited line among them exhibited elevated grain number per plant, grain weight per plant, panicle length, plant height, and Zn content relative to WT (Ludwig et al. 2024). The expression level of *OsNAS2* was found to be significantly elevated in certain promoter-edited lines (Ludwig et al. 2024). These results showed that editing the ARR1AT motif in the *OsNAS2* promoter enhanced Zn/Fe uptake

or translocation in rice (Ludwig et al. 2024). This study demonstrates the feasibility of regulating the uptake, translocation, and accumulation of micronutrients in rice through promoter editing. Additionally, it provides insights into fine-tuning the micronutrient content of rice to create more nutritious varieties in the future.

Vacuolar H^+ translocating pyrophosphatase (V-PPase) affects cytoplasmic pH and is an enzyme activity regulator that degrades starch to sucrose. The research showed that editing the GATA element in the promoter of the V-PPase gene significantly downregulated V-PPase and reduced rice chalkiness (Dharwadker et al. 2022; Gann et al. 2023), while this downregulation also reduced germination and slowed seedling growth. In addition, the lower germination rate and slower seedling growth were found in edited lines, which would be attributed to the limitation of sucrose formation (Dharwadker et al. 2022; Gann et al. 2023).

Future Perspectives

Promoter editing allows for precise gene regulation by inserting, deleting, or replacing specific bases or fragments within the CREs of genes. This approach enables the development of rice varieties with high yield, superior quality, and enhanced stress tolerance (Fig. 1). Although promoter editing can regulate gene expression in a different way from knocking out the coding region and achieve more subtle regulation of gene expression, it still has a certain degree of blindness and lacks perfect theoretical guidance. We identify several key issues with promoter editing and propose potential solutions to address these challenges.

Deeper understanding of promoter regulatory elements. Generally speaking, promoter editing needs to target promoter regulatory elements with specific functions, which are usually protein binding or recognition sites, but can also include conserved motifs or natural variation sites. The complexity of promoters includes numerous regulatory elements. While some elements have been elucidated through reliable experiments, many others remain with unknown functions. It is crucial to clarify the roles these elements play in regulating downstream genes and how they function. To achieve effective promoter editing, the targeted site must possess potential functionality rather than being an arbitrary sequence. This necessitates a well-designed, targeted approach rather than a blind one. As researchers gain a deeper understanding of the functions and mechanisms of nearly all regulatory elements, conserved motifs, and natural variant sites within gene promoters, promoter editing is expected to more precisely regulate gene expression. For instance, if a SNP in a gene promoter results in different spatial expression patterns across varieties, editing this SNP could potentially modify the gene's expression site. Similarly, if a conserved motif limits gene expression, inserting this motif into another gene could control its expression duration.

Gene editing systems are crucial tools for precise manipulation of genes. Since the inception of the first-generation gene editing system ZFNs, scientists have continually advanced the development of more refined systems, including TALENs, CRISPR/Cas9, CRISPR/Cas12, CRISPR/Cas13, Base Editing, and Prime Editing systems. While ZFNs and TALENs are now largely obsolete, CRISPR/Cas remains widely utilized. Of above systems, CRISPR/Cas9 has become the most widely used genome editing tool. However, each CRISPR/Cas system has its own features. We provided a comprehensive comparison of these CRISPR/Cas systems (Table 2). Although the CRISPR/Cas system has made significant strides, it still has limitations (Hussain & Ahmad 2022). Many current CRISPR/Cas systems depend on a sequence known as the protospacer adjacent motif (PAM) to bind or cut the target DNA. Targets lacking PAMs cannot be edited using traditional CRISPR/Cas systems, which significantly limits their flexibility. Many promoter regulatory elements or other desired editing sites may not offer adjacent PAMs, posing a considerable obstacle to precise

(See figure on next page.)

Fig. 1 Current applications and future perspectives for rice genetic improvement based on promoter editing. The future exploration of rice promoter CREs and functional motifs has the potential to broaden the scope of promoter editing and improve editing efficiency. Using advanced gene editing systems can also greatly improve the accuracy and efficiency of editing. In addition, it is possible to achieve precise control of gene spatiotemporal expression through promoter editing. This progress could lead to the development of an ideal rice variety with improved traits, such as higher yield, better quality, and greater stress resistance. **A** Potential targets for promoter editing, their possible effects on changing gene or protein expression levels or patterns, and future applications. In the future, more promoter regulatory elements should be discovered and elucidated, and advanced promoter editing systems should be used to transform them to ultimately achieve efficient rice improvement. **B** Modification results achievable by various editing strategies. Adapted from the published paper (Gao 2021). Fine editing promoter regulatory elements at the single-base level, inserting large functional fragments and promoter recombination or substitution may be the focus of future research. **C** Promoter editing has the potential to alter gene spatiotemporal expression patterns. One possible approach is to insert tissue- or time-specific promoter elements. Another is to inserting environmentally responsive promoter elements to induce or inhibit gene expression through exogenous treatments such as light, temperature, and chemical reagents
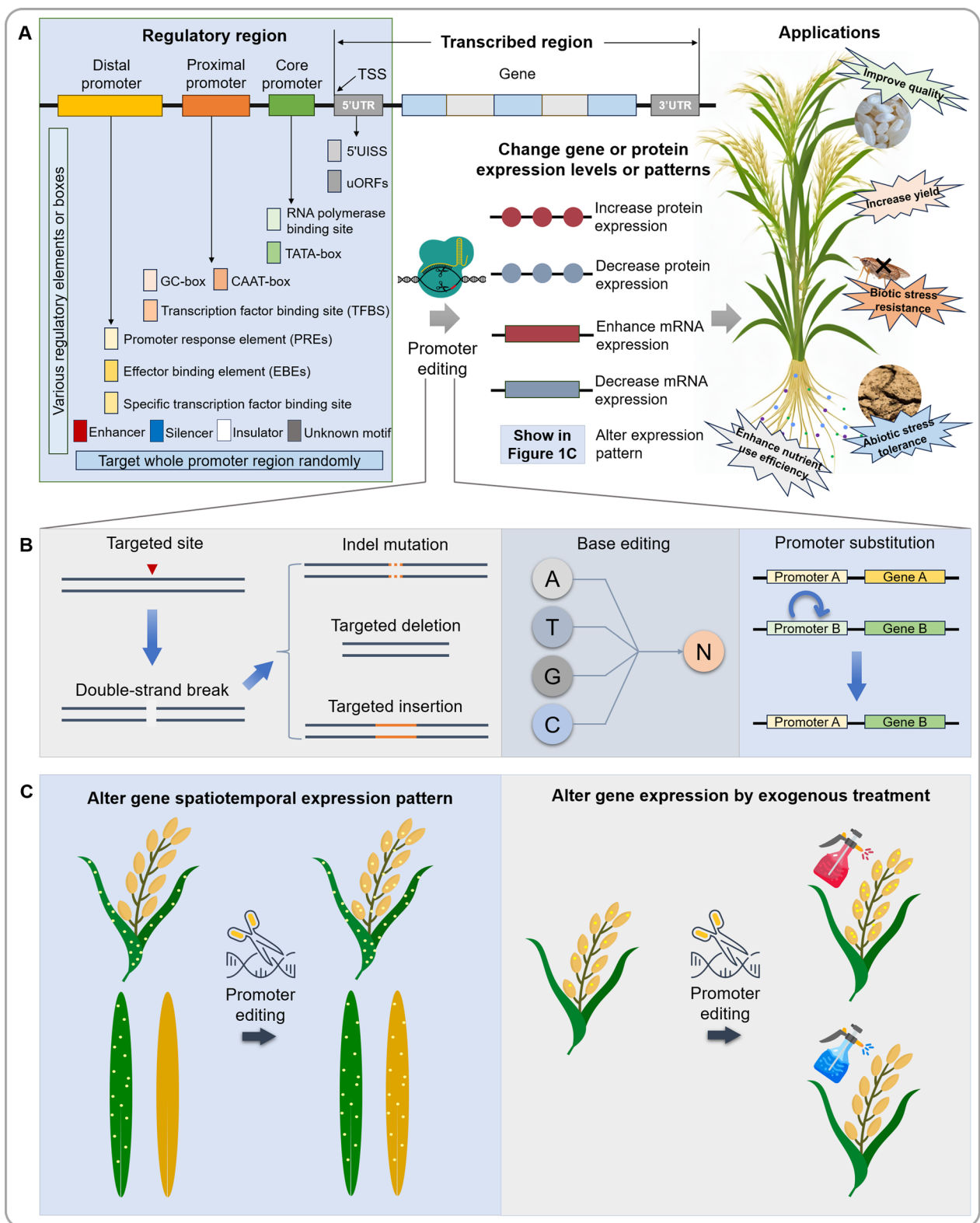
Fig. 1 (See legend on previous page.)

Table 2 Comparison of key features of CRISPR/Cas systems, focusing on promoter editing

Features						
CRISPR system	CRISPR/Cas3	CRISPR/Cas9	CRISPR/Cas12a (Cpf1)	CRISPR/Cas12b (C2c1)	CRISPR/CasΦ (PhiCas9)	CRISPR/CasX
Cas protein requirement	Cas3	Cas9	Cas12a (Cpf1)	Cas12b (C2c1)	CasΦ (PhiCas9)	CasX (CasIv)
Guide RNA requirement	crRNA	crRNA and tracrRNA	crRNA	crRNA and tracrRNA	crRNA	crRNA and tracrRNA
Cleavage pattern	Various	Blunt end	Sticky end	Sticky end	Sticky end	Blunt end
PAM requirement	A-T rich	5'-NGG-3'	5'-TTTV-3'	5'-TTN-3'	5'-TBN-3'	5'-TTCN-3'
Applicable vectors	Plasmid	Plasmid, viral vectors, ribonucleoprotein (RNP) complexes	Plasmid viral vectors, RNP complexes	Plasmid, viral vectors, RNP complexes	Plasmid, viral vectors, RNP complexes	Plasmid, RNP complexes
Common editing events	Large deletions, chromosomal rearrangements	Indels, point mutations, large deletions	Indels, base substitutions, targeted insertions	Indels, large deletions or replacements	Indels, targeted deletions	Indels, base substitutions
Advantages	Long-range DNA degradation	Well-studied, high precision, well-established protocols	Low off-target effects	High-temperature activity	Small size	Compact size
Multiplex editing efficiency	Undetermined	High	High	Moderate	Moderate	Undetermined
Current applications	Large deletions, etc	Knock-out, knock-in base editing, epigenetic modifications, etc	Knock-out, knock-in multiplexed editing, etc	Knock-out, knock-in, etc	Knock-out, epigenetic editing, etc	Knockout, etc
Application status in promoter editing	Undetermined, potential for long-scale editing or complete promoter deletion	The most mature system used in promoter editing	An effective system generating sticky ends, which is advantageous for precise gene insertion	Undetermined, its heat resistance may make it suitable for use in special environments	Undetermined, the small size of CasΦ protein may give it a distinct advantage in promoter editing	Undetermined, the small size of CasX protein may give it a distinct advantage in promoter editing

Various CRISPR/Cas systems have been developed for genome editing for the abundant Cas protein resources. The table comprehensively compares the key features, applications and potential for promoter editing of several CRISPR/Cas systems. At present, CRISPR/Cas9 system is still the most widely used promoter editing tool, which is especially true in rice promoter editing. However, it has shown that Cas12a system also has good application prospects. Nevertheless, other Cas systems may also have unique application potential in the field of promoter editing, which requires further study. In the table, N represents any nucleotides, V represents A, C, G, and B represents T, C, G

and flexible site-specific editing. To address these limitations, future efforts should focus on developing more efficient and versatile gene editing systems that either require a relaxed PAM sequence or eliminate the need for PAM altogether. Moreover, it is still not easy to accurately insert large fragments at desired sites and target specific bases for editing. To address these issues, the development of gene editing systems must be advanced simultaneously.

The precise regulation of rice growth and development has long been a challenge for breeders. By modifying rice promoters with advanced gene editing techniques, researchers can regulate gene expression within specific ranges, thus influencing rice traits. Future advancements may include the use of diverse gene editing systems to modify rice promoters, creating binding sites for specific transcription factors or DNA-binding proteins. One promising solution is the use of technologies based on CRISPR system to insert functional sites from identified tissue-specific promoters into the promoters of certain genes that are desired to express in specific parts of the body. This approach allows for the expression of genes in specific tissues without the risk of undesirable effects that can arise from using strong promoters that drive expression throughout the entire plant. By integrating key sites of inducible promoters into specific genes, it is also possible to control gene expression at precise times through exogenous induction. Similarly, replacing the promoters of particular genes with tandem tissue-specific and inducible promoters in vitro enables precise regulation of rice growth and development in targeted tissues and at specific developmental stages. This methodology offers a refined approach to managing gene expression, ensuring that desired traits are achieved without compromising other essential characteristics.

Targeting the coding sequence to generate gene mutations is undoubtedly the most direct way to alter phenotypes. However, even with knockouts, residual translation of non-functional proteins can occur, leading to wasted energy and potentially detrimental effects on rice growth and development, which can negatively impact agricultural production. In contrast, modifying promoters avoids the production of redundant proteins, making the resulting mutant lines safer and more efficient by eliminating the waste of energy on ineffective proteins. This approach also allows for precise regulation of gene expression and enables targeted gene knockdown without the lethal effects sometimes associated with complete gene knockouts or the instability of heterozygous knockouts. Given its distinctive advantages—such as straightforward control, high precision, and minimal disruption to the plant's genetic structure—promoter region editing is likely to become a mainstream breeding method in the future. This approach offers a promising avenue for rice breeding, providing a more efficient means of achieving desired traits while minimizing unintended effects.

Due to genetic linkage and pleiotropy, crop breeding often faces trade-offs between traits. For instance, in rice, enhancing panicle weight can negatively impact tiller number. This mutually restrictive trade-off is a significant bottleneck in conventional breeding techniques. Overcoming these trade-offs is crucial for developing new varieties that are high-yielding, high-quality, and widely resistant. Natural crop evolution involves limited genetic mutations, which constrains breeders' ability to develop high-yielding, disease-resistant, and high-quality crop seeds. Even when beneficial mutations are identified, traditional breeding methods are time-consuming and require continuous refinement each year. Promoters, which control the timing and level of gene expression, play a key role in the pleiotropy of genes, as gene expression is tightly regulated by these elements. By modifying specific elements of a promoter, it is possible to optimize the expression pattern of a target gene and its associated trade-offs, thereby achieving breeding goals that improve yield, quality, and resistance simultaneously. Promoter gene editing technology allows for fine-tuning of gene expression without deleting genes or diminishing their protein-coding ability. This approach offers greater flexibility in improving quantitative traits of crops. By using gene editing to create and select the most ideal variants, breeders can control gene activity more precisely. This not only accelerates crop improvement but also enhances predictability, enabling breeders to "customize" crops according to planting conditions and select the best variants to maximize yields.

Promoter editing, which falls under the broader category of gene editing, raises important issues related to regulation, safety, and risk. With the advent of gene editing technologies like CRISPR, the term "Genetically Modified Crops (GMC)" has become well-known. However, concerns about GMC persist, primarily due to perceived risks to human health, animals, and the environment. These concerns include potential harm from gene-edited components, the possibility of edited genes producing toxic proteins, the risk of resistance genes leading to resistant strains, and the potential creation of superweeds through genetic drift (Ahmad et al. 2021a, b). To address these concerns, GMC are subjected to rigorous risk, toxicity, and biosafety assessments. Many countries, especially those with advanced regulatory systems, have implemented laws and regulations to control GMC and ensure that consumers have the right to know if the food they purchase is genetically modified. For instance, China, the United States, and several European Union countries employ an end-product-based

regulatory framework, labeling GM-free products accordingly and monitoring GMC before commercialization. Promoter editing, which uses techniques like CRISPR to alter genetic material, also falls within the scope of GMC regulations. Given the relatively recent introduction of promoter-edited crops and their limited practical application, there may be gaps in the current regulations, safety, and risk assessments for these crops. Therefore, in-depth research is needed to develop efficient, GM-free editing strategies (He et al. 2022). Policymakers should engage in discussions with researchers to formulate appropriate policies, ensuring that promoter-edited crops can be developed safely and effectively for the benefit of humanity in the future.

Taken together, promoter editing allows precise gene regulation by altering specific bases or fragments, enabling the development of better rice varieties. Unlike coding region knockouts, it offers nuanced gene expression control but requires a deeper understanding of promoter elements. Advanced gene editing systems like CRISPR/Cas have improved precision but still face limitations. Promoter editing avoids redundant protein production, and helps balance genetic trade-offs in breeding. However, promoter-edited crops raise regulatory and safety concerns, necessitating rigorous assessments and effective policies to ensure safe and beneficial applications. With continued research and technological advancements, promoter editing has the potential to revolutionize rice and other crop improvement and sustainable agriculture.

Conclusion

The regulation of gene expression at the molecular level is a crucial aspect of modern molecular breeding strategies, with promoters playing a pivotal role in this regulation. Promoter editing offers several key advantages. First, precise gene regulation: promoter editing allows for the fine-tuning of gene expression, which can enhance important agronomic traits in rice. Second, broad range of regulation: promoter editing can regulate gene expression in a wide range, thus generating a series of continuous QTVs, which is conducive to enhancing the ecological adaptability of rice or meets the requirements of more situations. Third, specificity in response: editing specific promoter regulatory elements can alter the sensitivity of the promoter to specific inducers or repressors, thereby achieving specific effects. Nevertheless, rice promoter editing continues to face significant challenges. Identifying appropriate editing sites for more precise modifications remains a critical issue. Additionally, there is a need for more efficient and accurate gene editing systems. However, the potential for rice promoter editing in genetic

improvement is substantial. With an enhanced understanding of rice promoter regulatory elements and advancements in gene editing technology, promoter editing is expected to become a more prominent tool in rice genetic improvement. This progress will likely lead to the development of numerous superior rice varieties through promoter editing.

Abbreviations
AAC	Apparent amylose content
AC	Amylose content
BLB	Bacterial leaf blight
BLS	Bacterial leaf streak
CAPE	CRISPR/Cas12a promoter editing
CREs	Cis-regulatory elements
EBEs	Effector-binding elements
ECQ	Eating and cooking quality
GMC	Genetically modified crops
HMP	High-efficiency multiplex promoter-targeting
KRs	Key-regions
PAM	Protospacer adjacent motif
PE	Promoter editing
QTL	Quantitative trait locus
SNP	Single nucleotide polymorphism
TALE	Transcription-activator-like effectors
TSS	Transcription start site
WT	Wild-type
5′UTR	5′ Untranslated region
CMS	Cytoplasmic male sterility

Data Availability
No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate
Not applicable.

Consent for Publication
Not applicable.

Competing interests
The authors declare no competing interests.

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