CRISPR/Cas to enhance gene expression for crop trait improvement by editing miRNAs targets

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Highlight

Removal of inhibitory elements can be a powerful approach to enhance gene expression. We argue that gene editing of miRNA target sites is a promising strategy, yet largely unexplored for crops.

Keywords

CRISPR/Cas9, miRNA, posttranscription, gene editing, crop

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Gene editing tools, such as CRISPR/Cas9, is often thought as a mean to prevent gene expression. However, a more subtle yet powerful approach is the enhancement of gene expression by precise deletion of repressor sites within a gene of interest. Recent reports demonstrate that editing sequences required for posttranscriptional regulation can result in transcript that is more stable or more translated, with consequent trait improvement in crop plants. Whitin this approach, gene editing of miRNA target sites is remarkably promising because of the typical conservation of miRNAs and their targets, thus, likely ease to be translated from model to crop species. Here, we argue that gene editing of miRNA target sites is preferable over deletion of the miRNA genes themselves, given that it avoids the pitfalls of unspecific derepression of miRNA target(s) beyond the gene of interest.

Gene editing in crop improvement

Agricultural biotechnology is undergoing a revolution with the development of CRISPR/Cas gene editing tools (Jinek et al., 2012; Zaidi et al., 2020). There has been numerous reports of CRISPR-mediated genome edition in economically important crops (Zaidi et al., 2020), and cultivation and commercialization are already regulated in major world economies, such as in the United States, Canada, Brazil, Argentina, and Australia (Turnbull et al., 2021). It is likely that in a few more decades, gene edited food will be ubiquitous in most parts of the globe.

Gene editing using CRISPR/Cas, in its simplest form, is based on two components that come together to form a ribonucleoprotein (RNP) complex that can specifically recognize and cleave a target site within the genome. Such RNP complex is formed with an engineered noncoding RNA (i.e. clustered regularly interspaced short palindromic repeat; CRISPR) and a Cas protein (Jinek et al., 2012). In fact, today there is a vast diversity of CRISPR/Cas-based tools, as well as using different Cas proteins, allowing for a broad range of applications (Zaidi et al., 2020). Due to restrictions on genetically-modified crops (i.e. insertion of exogeneous genetic material), agricultural biotechnology has mostly benefited from CRISPR-mediated genome edition via transgenic expression of CRISPR/Cas, followed by transgene removal through plant crossing and genetic segregation, as well as via “transgene-free” approaches involving application of the RNP complex without plant genetic transformation (Chen et al., 2019). For example, preassembled CRISPR/Cas RNPs using Cas9 protein have been delivered using particle bombardment into maize and wheat embryos, and plants were regenerated without addition of exogenous genes (including no selection marker), resulting in on-target editing frequencies above 2% (Liang et al., 2017; Svitashev et al., 2016). Additionally, new developments and optimization of these protocols and systems, such as the use of miniature Cas protein that reduces the cargo to be delivered (Wu et al., 2021), will likely facilitate their adoption in crop improvement. Although the editing efficiency is still not impressive and the technique is laborious, delivery of preassembled CRISPR/Cas RNPs is the “cleanest” approach because no exogeneous DNA is ever introduced and, consequently, likely better perceived by the general public and more likely to receive regulatory approval.

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Gene editing beyond knockout

Currently, most edited crops have been engineered for gene knockout (i.e. loss of function), however, the advantages of using more subtle approaches are becoming apparent. Gene knockout using CRISPR/Cas9 has enabled crop trait improvement such as increased yield, improved quality, stress resistance, and breeding acceleration (hybrid breeding) (Chen et al., 2019). Although poorly explored yet, genome editing to modulate gene expression, as opposed to drastic inhibition, can enable fine-tuning of expression levels and distribution. For example, edition of regulatory genomic regions outside the gene of interest (i.e. *cis*-regulatory elements, such as promoter region) has been done in tomato (Rodríguez-Leal et al., 2017) and rice (Cui et al., 2020), leading to altered gene expression and phenotypical alterations. One advantage of this approach is the possibility to modify gene expression in a tissue-specific or developmental stage-specific manner, as well as to manipulate gene expression response to internal or external stimuli (Zhu et al., 2020). When introduction of exogenous gene is not undesirable, a catalytic inactive Cas enzyme (aka dCas) fused to transcriptional activators or repressors can be used to regulate gene expression via sequence-specific recruitment (provided by CRISPR sequence) of the dCas-transcriptional regulator fusion to a specific promoter (Pan et al., 2021).

Manipulation of posttranscriptional regulation using gene editing enables gene expression enhancement, usually via the removal of inhibitory elements that limit gene expression. Such inhibitory elements can be found within transcripts (e.g., sequence and structural features, and binding sites) and proteins (e.g., modification and catalytic sites, and interacting regions) (Liu et al., 2016; Roy and Arnim, 2013; Wray, 2003). Until recently, genome editing of posttranscriptional regulation applied to crop improvement has been limited to translational enhancement via targeting of upstream open reading frame (uORF). uORF is an example of transcript sequence feature in which a sequence upstream the main ORF encodes for a peptide, usually not conserved, and such unproductive translation reduces efficiency or inhibits ribosome from translating the main ORF. uORFs are commonly found in transcripts and edition of their start codon, as shown for LsGGP2 uORF using CRISPR/Cas9 in lettuce (*L. sativa*) (Zhang et al., 2018), can result in translation enhancement of the main ORF (e.g., LsGGP2) with corresponding trait improvement (e.g., increase in ascorbate content in lettuce). Sweeter strawberries (*F. vesca*) have also been produced by translation enhancement via uORF gene editing (Xing et al., 2020).

In plants, stability and translation of several transcripts are tightly regulated by specific miRNAs that display sequence complementarity to binding sites within target transcripts. miRNA binding sites and the miRNAs themselves are usually conserved, in contrast to other inhibitory elements such as uORF. miRNAs have also been targets of gene editing, and several reports have shown that CRISPR/Cas can be used to knockout specific miRNAs (recently reviewed by Deng et al., 2022), thus, enhancing target transcript gene expression with consequent trait improvement. However, this approach can result in pleiotropic or undesirable phenotype because all targets of the knocked-out miRNA will potentially be released from its inhibition. An alternative approach that is likely more specific has been recently

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shown for rice (*O. sativa*) improvement (Lin et al., 2021). The authors demonstrated that in-frame deletion of miR396 target site within *GROWTH REGULATING FACTOR 4* (*OsGRF4)* and *OsGRF8* coding sequence leads to enhanced transcript stability with consequent trait improvement (Fig. 1), i.e., larger rice grains and brown planthopper (BPH) resistance, respectively. Interestingly, even one single nucleotide deletion within the miRNA seed pairing (Box 1) was shown to effectively release a transcript from miRNA inhibition. Indeed, targeting the seed region is possibly the most robust approach to eliminate a miRNA repression because, although deletion of miRNA cleavage site is also likely to impact miRNA repression, removal of cleavage site might allow for residual miRNA translation inhibition to occur, given that its deletion does not prevent binding of miRNA-loaded argonaute complex (Box 1). In cases where in-frame deletion is detrimental to gene function, base editors and prime editors can be applied to introduce silent point mutations, without introduction nor removal of nucleotide, to rewrite the target sequence and, consequently, its regulation by a specific miRNA. Base editors have been used to introduce point mutation in miR156 targets in rice, but no evidence was shown for altered gene expression (Hua et al., 2019). Another possibility is when the target site is at an untranslated region (UTR), where editing has more flexibility. For example, the target site for miR156 at the 3' UTR of SQUAMOSA promoter-binding protein-like 13 (TaSPL13) in wheat was edited using CRISPR/Cas9, producing different indels, resulting in 2-fold increase in gene expression (Gupta et al., 2022). This tipped the trade-off between growth and yield in favour of increased wheat yield, with edited plants producing more and bigger grains. However, this strategy was limited to a single gene in the SPL gene family, because their target sequence for miR156 is found in the coding sequence in all transcripts, except for TaSPL13. As the CRISPR/Cas technology matures, gene editing will become more precise and predictable, for example with tools such as APOBEC-Cas9 (Wang et al., 2020), thus, broadening the miRNA regulatory networks amenable to gene editing. Gene editing applied to posttranscriptional regulation, including miRNA inhibition, is a powerful strategy in crop improvement that is still poorly explored.

Next steps in gene editing of posttranscriptional regulation

It is increasingly apparent that the path from gene to protein to function is far from orthogonal, given the myriad of regulatory mechanisms that modulates the abundance and activity of virtually all molecules found in a living organism. During transcription, modulation of processes that define the mature transcript sequence, such as transcription start site usage, polyadenylation signal and alternative splicing, are commonly associated with the presence of regulatory elements, without necessarily altering the encoded amino acid sequence. For instance, differential transcript start site usage modulates the presence of uORFs in response to light in the plant model Arabidopsis, by producing transcripts with shorter 5' UTR lacking inhibitory uORFs, thus, exhibiting enhanced translation (Kurihara et al., 2018). In fact, many regulatory processes previously thought to be independent from transcription occur cotranscriptionally, including RNA folding and RNA modification (Garcia-Lopez et al., 2018; Huang et al., 2019), blurring the line between transcriptional and posttranscriptional regulation. Once mature transcripts are released from the transcription site, regulatory elements found both in the transcript sequence and structure play essential role in delivering the genetic information, as well as in the regulation of other processes, such as RNA-

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dependent complex formation and phase separation. During translation, mRNA sequence might harbour distinct regulatory elements (e.g., uORF and miRNA target site), as well as less understood or unknown features that modulate RNA stability or translation efficiency, including particular RNA structures. Once translated, protein function is often tightly regulated through residue modifications (e.g., phosphorylation) and specific interactions (e.g., protein and metabolite). Furthermore, product of enzymatic activities can potentially vary with changes in enzyme composition, such as variation in the activity site. Although far more exhaustive, these various steps in which specific changes in the genome can potentially drive different biological functions, illustrate the broad and largely untapped diversity of CRISPR/Cas9 targets for crop improvement.

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Author contribution

RSR conceptualised the manuscript and wrote the first draft. SSF wrote part of the general gene editing section and revised the manuscript.

Conflict of interest

The authors declare no conflict of interest.

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Box

Box 1. Key elements in miRNA-target transcript base pairing and cleavage.

Most plant miRNAs are specific to a limited number of target transcripts, usually defined by perfect base pairing between miRNA seed region and target transcript (Brodersen and Voinnet, 2009). However, unperfect seed pairing is allowed in certain miRNA-transcript pairs, where base pairing with the miRNA 3' end compensates for the weak seed pairing. For instance, seed pairing in Arabidopsis miR398a-CDS2 exhibits one mismatch, while the remaining nucleotides exhibit only two mismatches.

Plant miRNAs guide transcript cleavage between their nucleotide 10 and 11. Therefore, mismatch in the cleavage site abolishes transcript cleavage that is usually readily quantified by RNA sequencing and quantitative PCR. However, cleavage-impaired miRNA-argonaute complex might still drive translation inhibition (Dugas and Bartel, 2008), and because translation regulation is rarely analysed in most works, phenotypical results from plants lacking perfect base pairing within cleavage site (e.g., via gene editing using CRISPR/Cas9) should be treated with caution.

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Figure legend

Figure 1. CRISPR/Cas9 deletion of miR396 target site in *OsGRF4*. (A) Position of miR396 target site in *OsGRF4* locus. (B) Detailed miR396 target site in *OsGRF4* transcript highlighting the miRNA seed pairing sequence and transcript cleavage site. Illustration of CRISPR/Cas9 target sequence used in (Lin et al., 2021). (C) Transcript comparison of wild type and *OsGRF4* in-frame deletions obtained in the same study. (B) and (C) show RNA nucleotide sequences and CRISPR/Cas9 target site is shown based on the corresponding genomic locus. Figure modified and adapted from (Lin et al., 2021).

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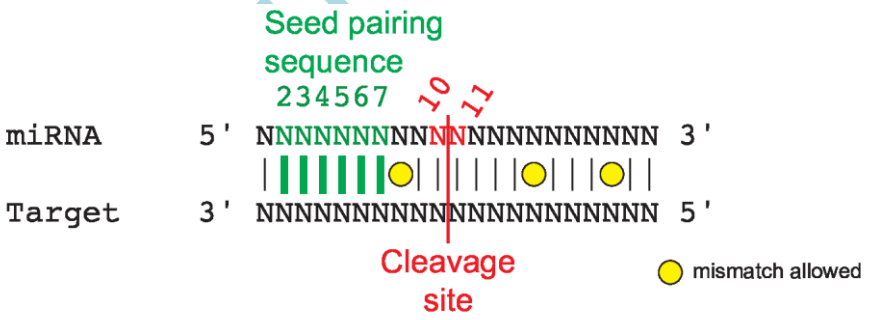
Box 1



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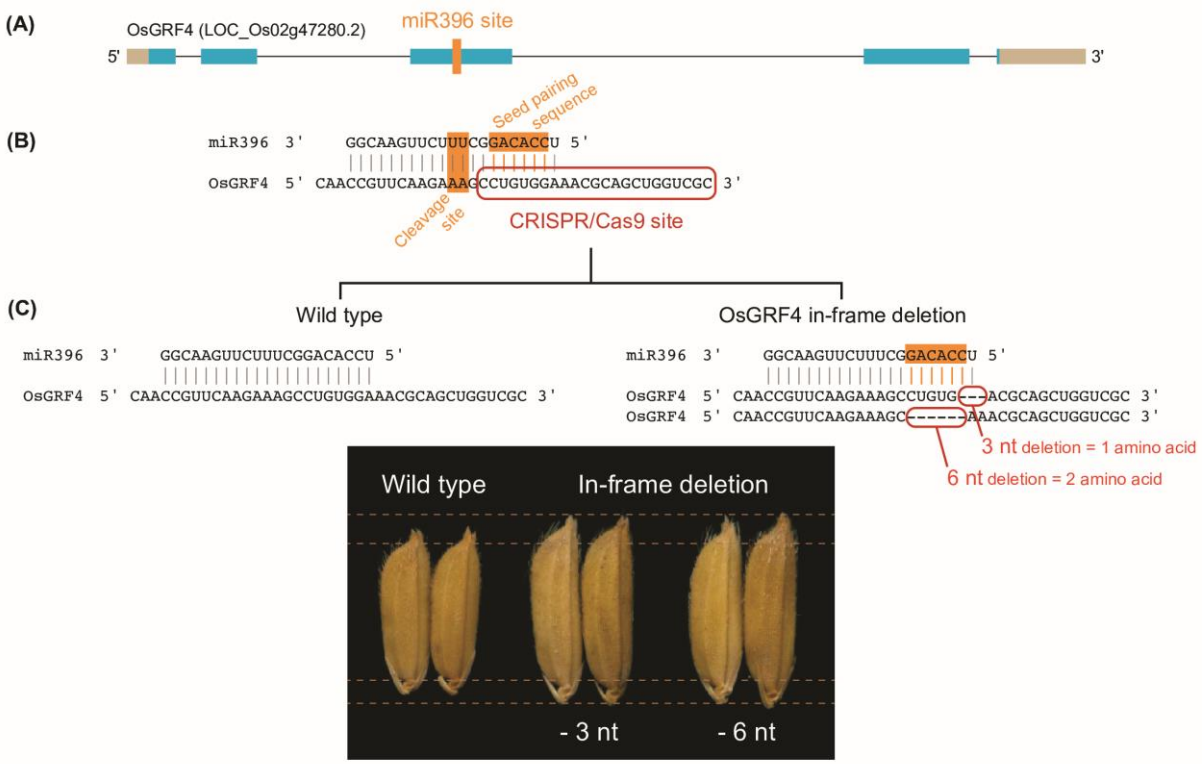

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