Engineered upstream open reading frames predictably downregulate mRNA translation in plants

Fine-tuning gene expression is crucial for generating quantitative phenotypic changes and crop improvement. Using CRISPR–Cas base editing and prime editing, we engineered upstream open reading frames — eukaryotic translational control elements — in rice to incrementally downregulate translation to predictable and desired levels.

Publisher's note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Published online: 09 March 2023

The question

The ability to control gene expression is essential for breeding new and desired traits into crops[1]. Widely used gene editing tools such as CRISPR–Cas, CRISPR interference and RNA interference generally prevent gene transcription completely or reduce it to a defined but unpredictable level[2]. CRISPR–Cas9-driven mutagenesis of promoters provides a method for generating quantitative phenotypic changes at the transcriptional level and producing a wide range of gene expression levels[3]. A method for predictably and incrementally downregulating endogenous gene expression at the translational level would expand on methods to control genes activity and benefit future crop improvements.

The solution

We have developed an efficient and tunable method for upregulating protein expression by knocking out endogenous upstream open reading frames (uORFs) on the gene mRNA[4]. uORFs are important and universal *cis*-regulatory elements in eukaryotes that are thought to be associated with reduced mRNA translation[5]. On the basis of our previous finding that disrupting uORFs can upregulate protein translation, we hypothesized that introducing de novo uORFs would have the opposite effect. Many factors, such as uORF length and the distance between the uORFs and the primary open reading frames (pORFs), affect the inhibitory activity of uORFs[5]. Thus, we thought that it should also be possible to reduce pORF translation by enhancing the inhibitory activity of existing uORFs. Furthermore, we thought that generating combinations of uORFs with different inhibitory activities might enable us to control the extent of downregulation of a given gene product (Fig. 1a).

To generate de novo uORFs, we introduced ATG start codons into 5′ untranslated regions (5′ UTRs) to produce uORFs encoding at least two amino acids (Fig. 1a). Similarly, to enhance the inhibitory ability of existing uORFs, we mutated their stop codons to lengthen their coding sequences while shortening intercistronic distances (Fig. 1a). uORFs were inserted upstream of the gene encoding firefly luciferase (LUC) in a dual-luciferase reporter system that also includes *Renilla reniformis* (sea pansy) luciferase (REN) as an internal control. Dual-luciferase and western blot assays in transient systems such as rice (*Oryza sativa*) protoplasts (plant cells stripped of cell walls) showed that the newly produced and the extended uORFs downregulated LUC/REN activity ratios to 9.5–86.9% of control levels but had no effect on LUC/REN mRNA levels, thereby providing proof of principle. In addition, using base editors or prime editors (CRISPR–Cas systems that introduce one single-base-pair change or small insertions and deletions, respectively), we obtained mutant rice plants carrying the new and extended uORFs and confirmed that the effects of these engineered uORFs on phenotypes and protein expression levels were the same as those observed in the protoplasts.

By combining these approaches, we were able to generate a suite of uORFs that incrementally downregulate the translation of pORFs to 2.5–84.9% of the wild-type level (Fig. 1a). Furthermore, by editing the 5′ UTR of rice *OsDLT*, which encodes protein DWARF AND LOW-TILLERING, a member of the GRAS family of transcriptional regulators involved in the brassinosteroid (BR) transduction pathway, we obtained a series of plants with varied BR sensitivity (Fig. 1b), plant heights and numbers of tillers (the grain-bearing branches) (Fig. 1c) that were consistent with the corresponding reductions in OsDLT levels observed in the protoplasts.

Future directions

In this study, we describe efficient and easy methods for downregulating protein translation to predictable and desired levels in plants by engineering uORFs. These methods enable the generation of genome-edited plants with graded expression of traits and, because uORFs are universal eukaryotic regulatory elements, should be applicable beyond the plant kingdom.

Because many factors, including secondary structure, downstream and upstream sequences and regulatory elements in the 5′ UTR, and the expression levels of translation initiation factors, among others, influence the inhibitory effects of uORFs[5], it is difficult to reliably predict the effects of uORFs on pORF translation and phenotypes. We have demonstrated that systems such as dual-luciferase reporter assays can rapidly and reliably predict the effects of uORFs. Nevertheless, detailed knowledge of how each factor influences the inhibitory effects of uORFs should enable more effective exploitation of these approaches. Next, we intend to apply these methods to improve crop traits by circumventing the complex trade-offs resulting from pleiotropy (the phenomenon of a single gene affecting multiple traits).

Chenxiao Xue & Caixia Gao

Institute of Genetics and Developmental Biology, Beijing, China.

EXPERT OPINION

"Trait phenotypes can be modulated by promoter editing on the transcriptional level. This work demonstrates that, by manipulation of upstream open reading frames, trait phenotypes can also be modulated on the translational level. Thus, we have now two independent levels of fine-tuning the expression of traits in crops with standard editing technology. This study presents overwhelming evidence that the approach is working and feasible, and it is definitely of general interest to a wide readership." **Holger Puchta, Karlsruhe Institute of Technology, Karlsruhe, Germany.**

FIGURE
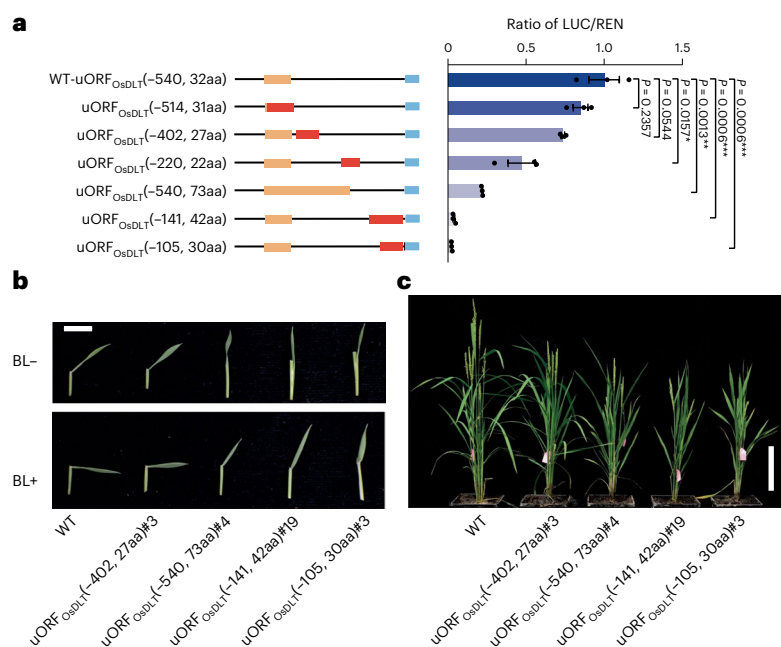
Fig. 1 | Breeding plants with a predicted phenotype by engineering uORFs. a, Schematic representation of engineered uORFs in the 5' UTR of *OsDLT* (left) and the effects of these uORFs on LUC/REN activity ratios (right) in dual-luciferase assays. The red, orange and blue squares represent new uORFs, endogenous uORFs and pORFs, respectively. The ($-X$, Yaa) annotations indicate that the uORF starts at position X nucleotides upstream of the pORF start codon and is Y amino acids long. Ratios are presented as mean ± s.e.m.; dots represent individual assays. WT, wild type. **b,c,** The lamina joint is the angle between the leaf blade and the sheath; BR promotes lamina inclination. BR sensitivity (**b**) and morphology (**c**) of WT plants and T_1 progenies of edited plants carrying the engineered uORFs. BL, epi-brassinolide. Scale bars, 1 cm (**b**) and 25 cm (**c**). © 2023, Xue, C. et al.

BEHIND THE PAPER

Obtaining genome-edited plants carrying the engineered uORFs was important to ensure that the methods that we developed in this work were applicable at the whole-plant level. However, when using base editors and prime editors to generate the desired edits, we found that the editing efficiencies of many prime editing guide RNA–prime editor combinations were very low. Fortunately, as part of another project in our laboratory, we had developed an engineered prime editor with a substantially improved editing efficiency in plants. Moreover, combining our engineered prime editor with a previously reported engineered prime editing guide RNA further increased editing efficiency. The use of this combination helped us to obtain enough mutants for further experiments. **C.X. & C.G.**

REFERENCES

1. Gao, C. Genome engineering for crop improvement and future agriculture. *Cell* **184**, 1621–1635 (2021).
   A review of the development and application of genome editing tools in plants.
2. Bowman, E. K. et al. Bidirectional titration of yeast gene expression using a pooled CRISPR guide RNA approach. *Proc. Natl Acad. Sci. USA* **117**, 18424–18430 (2020).
   A plasmid-based library methodology for bidirectional, graded modulation of gene expression in yeast.
3. Rodríguez-Leal, D. et al. Engineering quantitative trait variation for crop improvement by genome editing. *Cell* **171**, 470–480.e478 (2017).
   A way to dissect the biology of quantitative trait loci by targeting promoters using CRISPR–Cas9.
4. Zhang, H. et al. Genome editing of upstream open reading frames enables translational control in plants. *Nat. Biotechnol.* **36**, 894–898 (2018).
   A method for upregulating protein expression by editing uORFs.
5. Zhang, T., Wu, A., Yue, Y. & Zhao, Y. uORFs: important *cis*-regulatory elements in plants. *Int. J. Mol. Sci.* **21**, 6238 (2020).
   A review article describing the roles of uORFs in modulating plant growth, development and environmental adaptation.

FROM THE EDITOR

"There is a need for fine-tuned gene editing in plants. This group has previously looked at increasing gene expression by editing uORFs, but here they extend this approach to the downregulation of gene expression in rice, to produce a variety of graded phenotypic traits." **Editorial Team, *Nature Biotechnology***