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Manipulating gene translation in plants by CRISPR–Cas9-mediated genome editing of upstream open reading frames

Xiaomin Si[1,2], Huawei Zhang[1], Yanpeng Wang[1], Kunling Chen[1] and Caixia Gao[1,2*]

Gene expression is regulated by multiple processes, and the translation of mRNAs into proteins is an especially critical step. Upstream open reading frames (uORFs) are widespread *cis*-elements in eukaryotic genes that usually suppress the translation of downstream primary ORFs (pORFs). Here, we describe a protocol for fine-tuning gene translation in plants by editing endogenous uORFs with the CRISPR–Cas9 system. The method we present readily yields transgene-free *uorf* mutant offspring. We provide detailed protocols for predicting uORFs and testing their effects on downstream pORFs using a dual-luciferase reporter system, designing and constructing single guide RNA (sgRNA)–Cas9 vectors, identifying transgene-free *uorf* mutants, and finally comparing the mRNA, protein and phenotypic levels of target genes in *uorf* mutants and controls. Predicting uORFs and confirming their effects in protoplasts takes only 2–3 weeks, and transgene-free mutants with edited target uORFs controlling different levels of pORF translation can be obtained within 4 months. Unlike previous methods, our strategy achieves fine-tuning of gene translation in transgene-free derivatives, which accelerates the analysis of gene function and the improvement of crop traits.

Introduction

Gene expression and its control play fundamental roles in determining the phenotypic diversity of living organisms[1]. Artificial manipulation of gene expression is an important strategy for optimizing the economic traits of industrial organisms, livestock animals and crop plants. Although much previous research in plants has aimed to manipulate gene expression at the transcriptional level[2–6], the translation of mRNAs into proteins is another critical mechanism to control gene expression that provides a more immediate way to alter the cellular content of encoded proteins to maintain homeostasis[7]. There is increasing evidence that uORFs, with their translation start codons located in the 5′ leader sequences of pORFs, can influence the translation of pORFs[8–11]. Both canonical AUG and non-AUG start codons are common in uORFs[9]. Transcriptome- and genome-wide studies have shown that many eukaryotic genes have associated uORFs. For example, 49% of human, 44% of mouse and 65% of zebrafish pORFs have associated putative AUG-initiated uORFs, and the same is true of >30% of the transcripts in 11 plant species, including monocotyledons (*Zea*, *Oryza*, *Hordeum*, *Panicum* and *Saccharum*) and dicotyledons (*Arabidopsis*, *Brassica*, *Citrus*, *Gossypium*, *Nicotiana* and *Glycine*)[11,12].

Several past studies have demonstrated the pivotal importance of uORFs in repressing translation of downstream pORFs[9–12], but the roles of the vast majority of the uORFs revealed by computational analysis remain unknown. It has been reported that antisense oligonucleotides that target uORF sequences can increase translation of the corresponding downstream pORFs[13], and uORFs can be used to optimize the translation of plant immunity genes so as to achieve a fine balance between disease resistance and cell growth[14]. These findings illustrate the potential impact of uORFs in improving vital plant processes and valuable crop traits. Hence, the development of an efficient and straightforward method for altering the sequences of uORFs should aid in dissecting the roles of these elements and help researchers to increase the expression of the products of downstream pORFs at the translational level.

[1]State Key Laboratory of Plant Cell and Chromosome Engineering, Center for Genome Editing, Institute of Genetics and Developmental Biology, the Innovation Academy for Seed Design, Chinese Academy of Sciences, Beijing, China. [2]College of Advanced Agricultural Sciences, University of Chinese Academy of Sciences, Beijing, China. *e-mail: cxgao@genetics.ac.cn

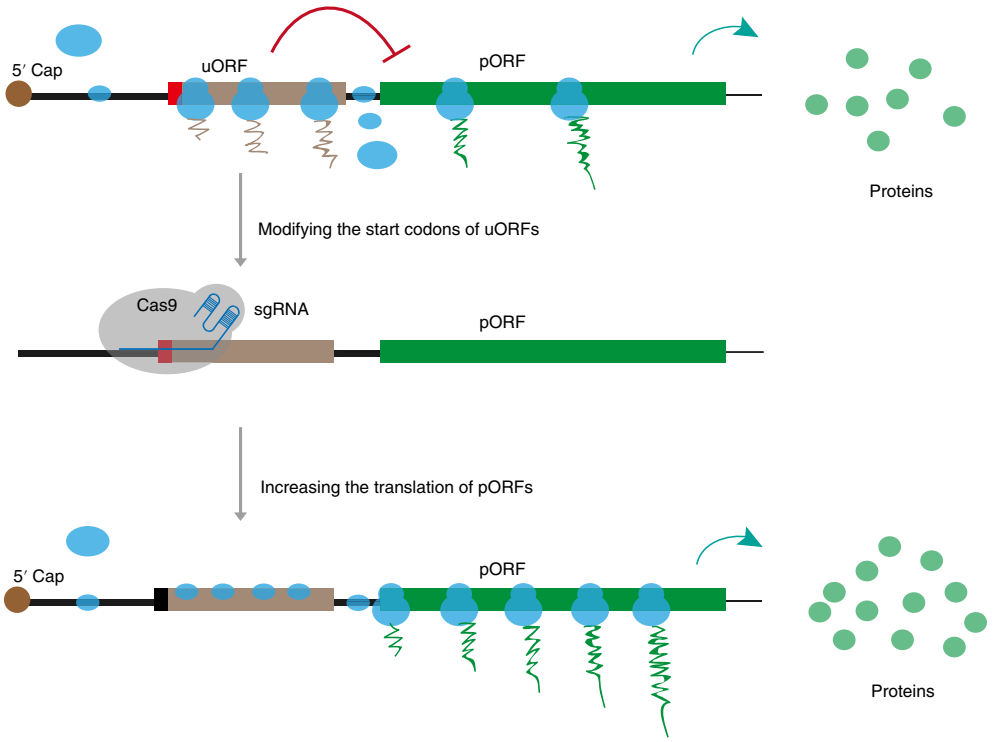

Fig. 1 | Schematic illustration of how gene translation is manipulated by editing upstream open reading frames. In eukaryotes, mRNA translation is initiated by a ribosome (blue) scanning an mRNA from its 5′ cap (dark brown). If it encounters the translation start codon (red) of a uORF (light brown), the uORF is translated and generally inhibits translation of the downstream pORF (green). By disrupting uORF regions with the CRISPR-Cas9 system, the inhibitory effects of uORFs can be prevented and translation of downstream pORFs enhanced. The small and large blue ovals represent the 40S and 60S ribosomal subunits, respectively. The zigzag lines associated with the 60S subunits represent the nascent polypeptide encoded by the uORF or pORF. The red line signifies inhibition, and the green circles represent proteins produced by pORFs.

The CRISPR–Cas9 system is a powerful and versatile genome-editing tool with applications in numerous organisms[15,16]. By generating double-strand breaks in target sites, it stimulates DNA repair by non-homologous end joining (NHEJ), which can disrupt genes by introducing nucleotide insertions and deletions. It can also induce the homology-directed repair (HDR) pathway, which can generate precise gene knock-ins and replacements[17]. The CRISPR–Cas9 system has been widely used to dissect gene functions and enhance plant traits[16]. Transgene-free mutant plants can be obtained by stably or transiently expressing CRISPR–Cas9 DNA or by using in vitro RNA transcripts or ribonucleoproteins (RNPs)[16,18–22]. In most cases, applications of the CRISPR–Cas9 system lead to loss of function of genes by targeting protein-coding sequences. However, for many important crop traits, one wishes to increase—rather than disrupt—gene expression.

Recently, we used the CRISPR–Cas9 system to introduce a range of nucleotide insertions and deletions into the uORFs of genes involved in development and antioxidant biosynthesis in *Arabidopsis thaliana*, lettuce and tomato, and we managed to fine-tune the translation of downstream pORFs in the *uorf* mutants[23,24] (Fig. 1). As genome-editing tools become increasingly versatile as a result of developments in base editing that permit single-nucleotide changes to be made efficiently[25–29] and incorporate the use of Cas9 variants with expanded protospacer-adjacent motif (PAM) recognition[16], manipulating uORFs will become progressively easier. The procedure described below provides an easy, efficient and generalizable method for fine-tuning the translation of important eukaryotic genes and should facilitate both basic and applied research in trait improvement.

Development of the protocol

uORFs are identified by searching for upstream translation start codons and in-frame termination codons upstream or downstream of the start codons of pORFs. A reliable dual-luciferase reporter system assay in protoplasts is then used to test the function of these putative uORFs. In the reporter system, the wild-type (WT) 5′ leader sequence, and the same sequence with its upstream translation

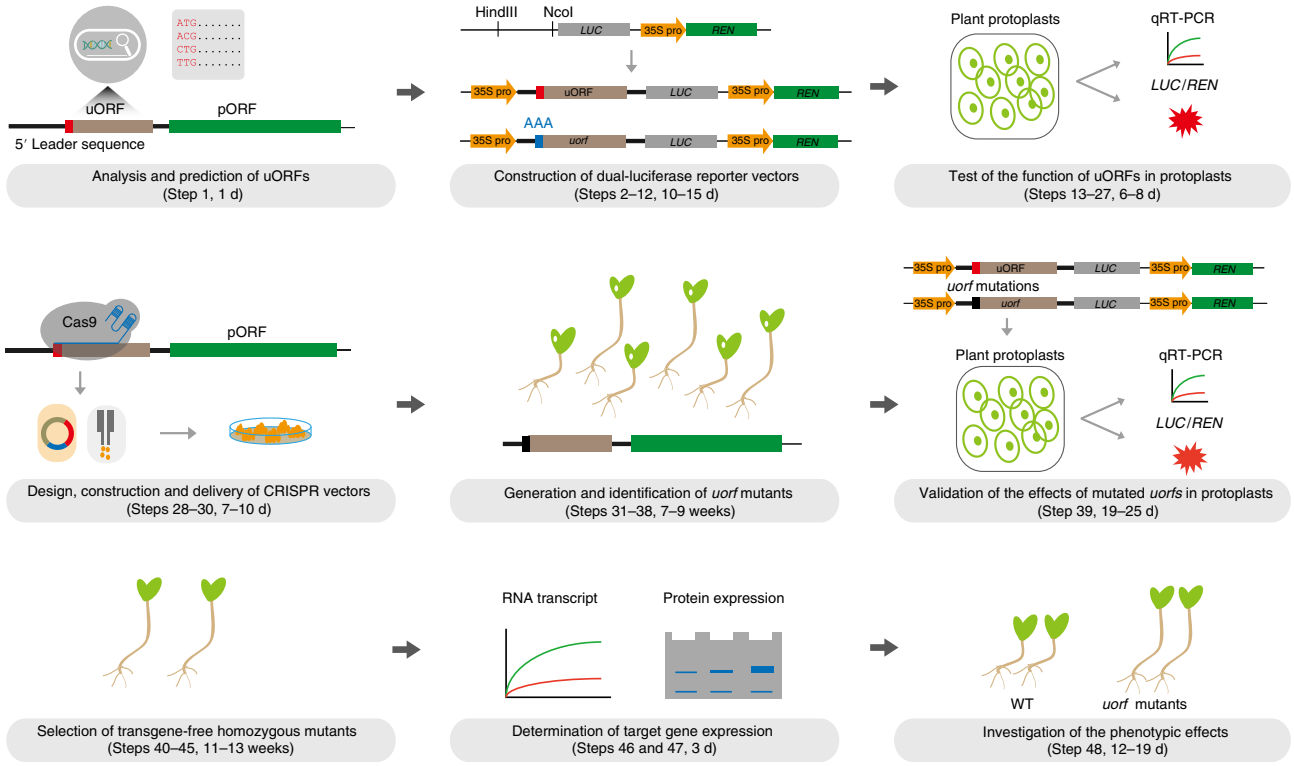

Fig. 2 | Overview of manipulation of gene translation by editing uORFs with the CRISPR-Cas9 system. *LUC*, luciferase gene; pro, promoter; *REN*, *Renilla* luciferase gene.

start codon inactivated, are separately positioned upstream of the firefly luciferase gene driven by the 35S promoter. If inactivating the upstream translation start codon enhances luminescence from the firefly luciferase reaction, a preliminary conclusion can be drawn that one has identified a functional uORF.

We then proceed to exploit this possibility, using CRISPR–Cas9 technology to generate plants with mutations around the start codon of the putative uORF. An sgRNA is designed to target the translation start codon of the putative uORF, with the cleavage site of Cas9 within the translation start codon or immediately next to it. Cleavage by CRISPR–Cas9 should then generate a set of alterations of the translation start codon and nearby sequences, some of which may influence translation of the uORF, giving rise to a series of mutants with different levels of translation of the downstream pORF.

Using PCR, we go on to identify transgene-free derivatives among the offspring and detect possible off-target effects in the mutants. Using qRT-PCR, western blotting and/or dual-luciferase reporter system assays, we examine the mRNA and protein levels of the target genes in these mutants and investigate the phenotypic effects of the variable expression of the target genes.

Overview of the procedure

The method presented here is an efficient and widely generalizable way to enhance gene translation in plants. The Procedure is illustrated in Fig. 2 and essentially comprises six stages: analysis and prediction of uORFs (Step 1), testing of the roles of the predicted uORFs in protoplasts (Steps 2–27), use of sgRNA–Cas9 vectors to target the uORFs and identify *uorf* mutants (Steps 28–38), validation of the function of mutated *uorfs* in transient assays (Step 39), screening of transgene-free and homozygous *uorf* mutants and analysis of off-target effects (Steps 40–45), estimation of transcript and protein abundance in these mutants and examination of phenotypes (Steps 46–48).

Applications of the method

Despite the widespread presence of putative uORFs in eukaryotic mRNAs, knowledge of their functions remains limited. Targeted modification of uORFs by our approach offers an opportunity to better understand their functions in translational control. In addition, it provides an alternative way to enhance endogenous gene expression. In some cases, the mutants that are generated have desirable

traits that can be used in crop improvement in place of routine transgene-generated overexpressing plants. By modifying the translation start codons of uORFs, or their contexts, the strength of repression exerted by them on the pORFs can be adjusted to different levels. Thus, our approach provides a powerful tool for fine-tuning the translation of downstream pORFs in plants.

Comparison with previous methods for enhancing gene expression

Conventional approaches used to enhance gene expression include transgene-mediated overexpression of exogenous genes[2–4], catalytically inactive Cas9 (dCas9)/DNase-deactivated Cpf1 (dCpf1)-mediated activation of endogenous genes[5,30] and CRISPR–Cas9-mediated modification of promoter regions[6]. Although these strategies are efficient and powerful ways of enhancing the expression of target genes, problems still restrict their application. Transgene-mediated overexpression of cDNA can substantially enhance gene expression, but transgene silencing usually occurs in the offspring plants[31], and random insertion of transfer DNA (T-DNA) may alter the normal sequence around the T-DNA insertion site[32]. In addition, this strategy is challenging and inefficient for enhancing the expression of multiple genes[33]; the same limitations apply to the use of dCas9-mediated activation of endogenous genes, although it can achieve multiplex gene activation[33]. Moreover, although the strategy of CRISPR–Cas9-mediated modification of promoter regions can generate diverse *cis*-regulatory alleles with varying gene expression levels[6], identifying such regulatory elements is difficult and time consuming. Our protocol has several unique advantages over the above three strategies for enhancing gene expression. First, it avoids the problem of gene silencing because uORFs are part of the stably inherited genome. Second, it has little effect on transcriptional patterns; in conventional transgenesis, genes of interest are driven by constitutive, tissue-specific or inducible promoters, none of which retain the exact native patterns of expression of the relevant endogenous genes, and such changes can have negative effects on plant growth and development or generate abnormal phenotypes[34–36]. Because uORFs are *cis*-elements that suppress translation of pORFs, and the pORF promoters are unchanged in our approach, most uORF-edited mutations should not substantially alter transcription of the pORFs but only affect their translation. Third, it is easy to predict and verify uORFs; the 5′ leader sequences of mRNAs are usually much shorter than the promoter regions and can be found in public genome databases such as that of the National Center for Biotechnology Information (NCBI). Prediction of uORFs is based on identifying translation start codons in 5′ leader sequences; usually, several putative start codons exist in this region and a dual-luciferase reporter system is used to quickly check the potential uORFs in protoplasts assays. Thus, prediction and verification of uORFs are very easy. Although similar outcomes can be achieved by mutating negative regulatory promoter elements[6], identification of such elements is difficult. Fourth, traditional transgene overexpression based on insertion of foreign DNA is a central biosecurity issue that arouses public concern; our method enhances endogenous gene expression without integration of exogenous DNA, thus making it more acceptable to the public and regulatory authorities. Moreover, the delivery of the CRISPR–Cas9 system by RNPs, a recently developed method, yields exogenous DNA-free mutants in the T_0 generation without the need for further manipulation[20–22]. In summary, our protocol fine-tunes endogenous gene expression by introducing mutations into the uORFs of target genes, and these changes are stably inherited. More importantly, it yields transgene-free progeny and thus accelerates the dissection of gene function and the improvement of crop traits. Current advances in precise gene targeting, such as base editing[25–29], which generates targeted single-base changes without DNA double-strand cleavage or the use of a donor template, make it more efficient to use base editors for precisely modifying uORFs, as is done in our approach.

Limitations of this approach

Limited range of application

Our approach can be used only for mRNAs with functional uORFs. Although uORFs are widespread, some genes do not contain the canonical translation start codon of upstream AUG (uAUG), which is correlated with strong repression of translation of the corresponding pORFs.

Restricted strengths of the repression by uORFs

Compared with transgene-mediated overexpression, editing of a uORF usually results in only a modest increase in gene expression level that is related to the strength of the translational repression of the pORFs exerted by the uORFs.

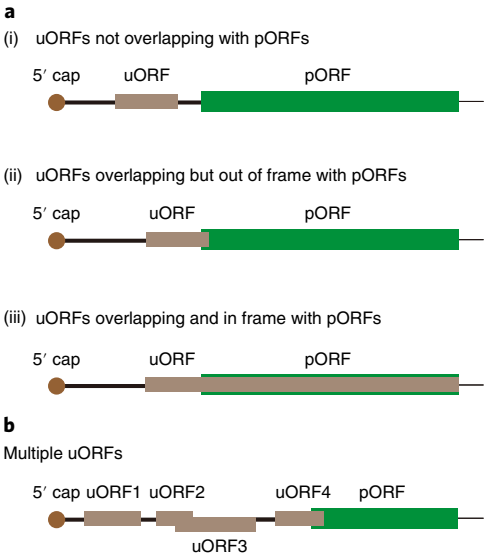

Fig. 3 | Categories of uORFs according to the positions of their start codons and stop codons. a, Types of uORFs. **b**, Multiple uORFs.

PAM sequences

The CRISPR–Cas9 system can function only with 5′-NGG or 5′-NAG PAMs, which limits its target range. However, engineered variants of Cas9 and novel CRISPR–Cas systems permit a wider choice of PAM sequences.

Experimental design

Here, we present the detailed steps we use to enhance the level of translation of uORF-containing genes in *A. thaliana*, lettuce, and tomato; these procedures also apply to other genes in different plant species.

Prediction of uORFs

uORFs are short ORFs located in the 5′ leader sequence of eukaryotic genes that are defined by a start codon that terminates with an in-frame stop codon that precedes or follows the start codon of a pORF. To predict a uORF, we first search for the 5′ leader sequence of the relevant target gene in a genome database such as Phytozome v.12.1 (https://phytozome.jgi.doe.gov/pz/portal.html) or NCBI (https://www.ncbi.nlm.nih.gov/). Prediction of a putative uORF is based on the following criteria.

Start codon and stop codon. AUG is the major form of start codon for uORFs. Non-canonical translation start codons deviating from the canonical start codon (AUG) by one nucleotide (such as ACG, CUG, UUG) are also present in some uORFs[9,37]. The presence of a Kozak consensus sequence (with a purine at the −3 position and a G at the +4 position) is correlated with the initiation efficiency at a non-canonical start codon or canonical AUG codon[37,38]. The stop codons of uORFs can be UAA, UGA or UAG.

Length. We define uORFs as consisting of at least 9 nt, including a start codon and a stop codon.

Location and number. In general, uORFs can be divided into three types according to their positions: (i) uORFs not overlapping with pORFs (the stop codons of the uORFs are situated entirely upstream of the start codons of the pORFs); (ii) uORFs overlapping but out of frame with pORFs (the stop codons of the uORFs overlap with the start codons of the pORFs but are in different reading frames); and (iii) uORFs overlapping and in frame with pORFs (they share the same stop codons)[9,10] (Fig. 3a). A given pORF may be preceded by more than one uORF, and the uORFs may be separated or overlap in frame or out of frame[10,11] (Fig. 3b). In plants, 30% of mRNAs have uAUGs and 5% have three or even more[8]. Usually, the extent of inhibition of translation of a pORF is a function of the number of uORFs[39].

Homology. Conserved peptide uORFs (CPuORFs), special uORFs that encode conserved peptides, occur in <1% of plant mRNAs and are conserved in different species and gene families[39,40]. They are important for inhibiting translation of pORFs and are appropriate targets for manipulating gene translation. To check if candidate uORFs are CPuORFs, we align them with DNA sequence or amino

acid BLAST in NCBI or Phytozome v.12.1 to see whether they are conserved in gene families and/or in different species. The uORF of *AtBRI1* described in this protocol is canonical, whereas the uORF of *LsGGP1* is a CPuORF conserved in different species.

Testing the functions of uORFs in protoplasts

To examine the effect of a putative uORF, we see whether artificially mutated forms of the uORF alter translation of the downstream protein-coding mRNA in transient assays using a dual-luciferase reporter system[41]. The reporter vector pGreenII0800-LUC consists of two reporter genes; one is a promoter-less firefly luciferase (*LUC*) gene with multiple upstream cloning sites and the *Cauliflower mosaic virus* (CaMV)-terminator, and the other is the *Renilla* luciferase (*REN*) reporter regulated by the 35S promoter and CaMV-terminator; this serves as an internal control. The 5′ leader sequence containing candidate or artificially mutated uORFs (the putative start codon of the uORF is mutated to AAA to prevent translation of the putative uORF) driven by the 35S promoter is cloned upstream of *LUC*, and changes of *LUC* translation (or transcription) reflect the candidate uORF's influence on the pORF. For transient assays in protoplasts, 20 μg of plasmid DNA and ~5×10^5 protoplasts are used for each reaction. Methods for isolating and transfecting protoplasts vary according to the species. After incubation for 2 d, the protoplasts are harvested and used for measuring LUC/REN activities and mRNA levels. The protoplast transformation efficiency should not be <50%, and at least five independent biological replicates are advised to avoid random error.

Designing sgRNAs and disrupting uORFs with the CRISPR–Cas9 system

Different *cis*-elements in mRNA 5′ leader sequences act as key elements regulating translation initiation[9] and deleting the entire uORF may have unexpected effects on regulation of the pORF. Thus, to reduce translation of a uORF directly and effectively, we design one sgRNA to destroy the start codon or the sequence around it. By using this strategy, we aim to create a set of *uorf* mutants that differ in the efficiency with which they relieve the inhibition of pORF translation and so provide opportunities for fine-tuning of a biological trait. We use different constructs to express the CRISPR–Cas9 system in different plants. For example, in *Arabidopsis*, we use the *YAO* promoter-driven CRISPR–Cas9, which is preferentially expressed in tissues undergoing cell division and highly expressed in the embryo sac, embryo, endosperm and pollen, so that endogenous genes can be efficiently modified[42]. However, sometimes the potential target regions of the CRISPR–Cas9 system are limited by its PAM. In such cases, other CRISPR–Cas nucleases (e.g., *SaCas9* and Cpf1) and engineered Cas9 variants (e.g., SpCas9-NG and xCas9) can be used to broaden the choice of PAM[43–46].

Identifying *uorf* mutants, and identifying transgene-free mutants in the offspring

Transformations of *Arabidopsis* and lettuce have been previously described[23,47], as have procedures for identifying CRISPR-mediated mutants[48,49]; these include PCR/RE, T7EI cleavage assay, Sanger sequencing and next-generation sequencing (NGS). After obtaining a set of *uorf* mutants, the transient dual-luciferase reporter system is used to examine the influence of the mutated *uorfs* on the corresponding pORFs at the transcriptional and translational levels. Lines with functional *uorf* mutations validated by transient assay are selected for further analysis. To obtain transgene-free mutants, the seeds of T_1 *Arabidopsis* and T_0 lettuce are harvested to obtain transgene-free mutants in the next generation. We then design several pairs of primers covering the sgRNA–Cas9 expression construct to amplify transgene fragments and identify plants whose genomic DNA fails to be amplified with these primers. For *Arabidopsis*, we also sow the seeds in 1/2 Murashige & Skoog (MS) medium containing antibiotic that inhibits the growth of transgene-free mutants.

Analysis of off-target effects in transgene-free mutants

To assess off-target effects of the CRISPR–Cas9 system, we identify potential off-target sites of the sgRNAs using the Cas-OFFinder software (http://www.rgenome.net/cas-offinder/)[50]. The most likely sites are selected and off-target effects are analyzed with the T7EI assay.

Measuring gene expression in the mutants at the RNA, protein and phenotypic levels

mRNA levels are quantified by qRT-PCR, and western blotting is used to measure protein levels. At the same time, we further examine gene expression indirectly by observing phenotypes. For example, in *Arabidopsis*, the overexpression of *AtBRI1* reduces inhibition of *Arabidopsis* hypocotyl growth by brassinazole, a chemical that inhibits brassinosteroid (BR) biosynthesis[23].

Controls

For the transient assay in protoplasts, the 5′ leader sequence plus WT uORF is regarded as a control for the leader sequences containing mutated *uorfs*. For confirming the effects of modified *uorfs* on pORFs at the RNA, protein and phenotypic levels, WT plants or unmodified plants containing the transformation vector without sgRNAs serve as controls. For off-target effects, WT plants serve as controls.

Materials

Biological materials

- *Arabidopsis thaliana* (Columbia-0, available from the authors upon request)
- *Agrobacterium tumefaciens* EHA105 plasmid for *Arabidopsis* and lettuce (TransGen Biotech)
- Iceberg lettuce (*Lactuca sativa L.* var. *capitata*, available from the authors upon request)
- Tomato (*Solanum lycopersicum* cv. M82, available from the authors upon request)
- Chemically competent *Escherichia coli* DH5α cells (TransGen Biotech, cat. no. CD201-02)

Reagents

Plasmids and primers

- *Agrobacterium tumefaciens* EHA105 for *Arabidopsis* and lettuce (TransGen Biotech)
- pGreenII0800-LUC (Youbio Biological Technology, plasmid no. VT8124, http://www.youbio.cn/product/vt8124), for the dual-luciferase reporter system in protoplasts[41]
- AtU6-26-sgRNA-SK and pCAMBIA1300-*pYAO*:Cas9, for sgRNAs and Cas9 expression in *A. thaliana*[42]. These plasmids can be obtained from the Q. Xie laboratory (http://sourcedb.genetics.cas.cn/zw/zjrck/200907/t20090721_2130985.html) in the Institute of Genetics and Developmental Biology, Chinese Academy of Science
- pKSE401 (Addgene, plasmid no. 62202), for sgRNA and Cas9 expression in lettuce[51]
- pUC57 (GenScript, plasmid no. SD1176), for cloning the synthesized sequences
- PCR primers (BGI, custom order; https://www.genomics.cn/) are listed in Table 1 and in Supplementary Table 1
- qRT-PCR primers (BGI, custom order; https://www.genomics.cn/) are listed in Table 1

General reagents

- HindIII (Fermentas/Thermo Fisher Scientific, cat. no. FD0504)
- NcoI (Fermentas/Thermo Fisher Scientific, cat. no. FD0573)
- FastDigest Green Buffer (10×; Fermentas/Thermo Fisher Scientific, cat. no. B72)
- TAE buffer (10×; Cellgro, cat. no. 46-010-CM)
- Agarose (Invitrogen, cat. no. 16500500)
- AxyPrep DNA Gel Extraction Kit (Axygen, cat. no. AP-GX-250)
- T4 DNA ligase (Fermentas/Thermo Fisher Scientific, cat. no. EL0011)
- Taq MasterMix (2×; CWBIO, cat. no. CW0960A)
- Plasmid Miniprep Kit (Axygen, cat. no. AP-MN-P-250)
- Wizard Plus Midipreps DNA Purification System (Promega, cat. no. A7640)
- ClonExpressII One Step Cloning Kit (Vazyme, cat. no. C112-01)
- Glycerol (Amresco, cat. no. 0854)
- BsaI (New England BioLabs, cat. no. R0535)
- NheI (Fermentas/Thermo Fisher Scientific, cat. no. FD0973)
- SpeI (Fermentas/Thermo Fisher Scientific, cat. no. FD1254)
- Calf intestinal alkaline phosphatase (CIAP; Invitrogen, cat. no. 18009-027)
- Yeast extract (Sigma-Aldrich, cat. no. Y1625)
- Tryptone (Sigma-Aldrich, cat. no. T7293)
- ddH_2O (Novoprotein, cat. no. E130-01B)
- KOH (Sigma-Aldrich, cat. no. P5958)
- LB agar (Sigma-Aldrich, cat. no. A1296)
- NaOH (Sigma-Aldrich, cat. no. 795429)
- KNO_3 (Sigma-Aldrich, cat. no. P8291)
- $MgSO_4$ (Sigma-Aldrich, cat. no. M7506)
- $Fe_2(SO_4)_3{\cdot}6H_2O$ (Sigma-Aldrich, cat. no. 307718)
- KI (Sigma-Aldrich, cat. no. 746428)

Table 1 | Primer sequences used in this protocol

Step	Name	Sequence (5′–3′)	Purpose
8	pGreenII0800-LUC-F	TGCAAGGCGATTAAGTTGGGT	Forward primer for verifying positive clones of inserted fragment into pGreenII0800-LUC in colony PCR
8	pGreenII0800-LUC-R	CAGGAACCAGGGCGTATCTCTTC	Reverse primer for verifying positive clones of inserted fragment into pGreenII0800-LUC in colony PCR
11	pGreenII0800-LUC-F2	GAAGATGCACCTGATGAAATGGG	Forward primer for sequencing the inserted fragment into pGreenII0800-LUC
26	qPCR-LUC-F	GGATTACAAGATTCAAAGTGCG	Forward primer to quantify expression levels of *LUC* gene in qRT-PCR
26	qPCR-LUC-R	TGATACCTGGCAGATGGAAC	Reverse primer to quantify expression levels of *LUC* gene in qRT-PCR
26	qPCR-REN-F	CATGGGATGAATGGCCTGATATTG	Forward primer to quantify expression levels of *Ren* gene in qRT-PCR
26	qPCR-REN-R	GATAATGTTGGACGACGAACTTC	Reverse primer to quantify expression levels of *Ren* gene in qRT-PCR
Box 1, step 1A(vii)	At-gRNA-F	CTCACTATAGGGCGAATTGG	Forward primer for colony PCR and sequencing
Box 1, step 1A(x)	1300-sgRNA-F	CCAGTCACGACGTTGTAAAAC	Forward primer for colony PCR and sequencing
Box 1, step 1A(x)	1300-sgRNA-R	CAATGAATTTCCCATCGTCGAG	Reverse primer for colony PCR
Box 1, step 1B(i)	pKSE401-U6-F	CCAGTGCCAAGCTTCGACTTG	Forward primer to sequence for sgRNA vector verification
33	BRI1-F	AAGTAGGATATGTAGCTTGCAGAAG	Forward primer to amplify the $uORF_{AtBRI1}$ target site
33	BRI1-R	AGATCCAGAACTTCCAAGCTG	Reverse primer to amplify the $uORF_{AtBRI1}$ target site
33	LsGGP1-F	TCGAATTAATTTGCGACTAGC	Forward primer to amplify the $uORF_{LsGGP1}$ target site
33	LsGGP1-R	CTTCTTCGATTAATTGGGACGC	Reverse primer to amplify the $uORF_{LsGGP1}$ target site
33	M13F	CAGGAAACAGCTATGACC	Forward primer to sequence the clones
33	M13R	TGTAAAACGACGGCCAGT	Reverse primer to sequence the clones
42	At-Yao-crispr--F1	CCAGTCACGACGTTGTAAAAC	Forward primer to detect CRISPR-Cas9 construct in *Arabidopsis*
42	At-Yao-crispr--R1	CAATGAATTTCCCATCGTCGAG	Reverse primer to detect CRISPR-Cas9 construct in *Arabidopsis*
42	At-Yao-crispr--F2	CTCGAGGAAGCTTCTAGATTTC	Forward primer to detect CRISPR/Cas9 construct in *Arabidopsis*
42	At-Yao-crispr--R2	GATCCTTGTAGTCTCCGTCGTGG	Reverse primer to detect CRISPR-Cas9 construct in *Arabidopsis*
42	At-Yao-crispr-F3	CATCCAGAAAGCCCAGGTGTC	Forward primer to detect CRISPR-Cas9 construct in *Arabidopsis*
42	At-Yao-crispr--R3	CAGGGTAATCTCGGTCTTG	Reverse primer to detect CRISPR-Cas9 construct in *Arabidopsis*
42	At-Yao-crispr-F4	CATCATGGAAAGAAGCAGCTT	Forward primer to detect CRISPR-Cas9 construct in *Arabidopsis*
42	At-Yao-crispr--R4	GAATTCCCGATCTAGTAACATAGA	Reverse primer to detect CRISPR-Cas9 construct in *Arabidopsis*
42	At-crispr-control-F5	AGTTACTTCGATTGATCTCAGCT	Forward primer to amplify the fragment of *AtBRI1* in *Arabidopsis*
42	At-crispr-control-R5	GAGAGATCGAGACCAGTGAGTG	Reverse primer to amplify the fragment of *AtBRI1* in *Arabidopsis*
42	Ls-pKSE401-F1	GAAACGACAATCTGATCCAAGCTCAAGC	Forward primer to detect CRISPR-Cas9 construct in lettuce
42	Ls-pKSE401-R1	CAATCACTACTTCGACTCTAGCTGTATA	Reverse primer to detect CRISPR-Cas9 construct in lettuce
42	Ls-pKSE401-F2	GCAGTTCCGATGAGATAAACCAATAAGC	Forward primer to detect CRISPR-Cas9 construct in lettuce

Table continued

Table 1 (continued)

Step	Name	Sequence (5′–3′)	Purpose
42	Ls-pKSE401-R2	AGAAGTAGAATGCTTGATTGCTTGAG	Reverse primer to detect CRISPR–Cas9 construct in lettuce
42	Ls-pKSE401-F3	GATTACAAGGATGATGATGACAAGATGGCT	Forward primer to detect CRISPR–Cas9 construct in lettuce
42	Ls-pKSE401-R3	GTTATCCAGGTCATCGTCGTATGTGTCCTT	Reverse primer to detect CRISPR–Cas9 construct in lettuce
42	Ls-pKSE401-F4	GGACATTGTGCTGACACTCACTCTGTTC	Forward primer to detect CRISPR–Cas9 construct in lettuce
42	Ls-pKSE401-R4	GCTGGCGCCAGTAGTTCTTCATCTTCTT	Reverse primer to detect CRISPR–Cas9 construct in lettuce
42	Ls-crispr-control-F5	CTGTTTCGCTATGATGTTACTGC	Forward primer to amplify the fragment of *LsGGP1* in lettuce
42	Ls-crispr-control-R5	CATGCATGAATCAGAAACAACGT	Reverse primer to amplify the fragment of *LsGGP1* in lettuce
46	qPCR-AtBRI1-F	TTGGTTCTTGCTCCGGTCTG	Forward primer to quantify expression levels of target gene *AtBRI1* in *Arabidopsis*
46	qPCR-AtBRI1-R	CGTCTCCACTGATTTTGTTTCC	Reverse primer to quantify expression levels of target gene *AtBRI1* in *Arabidopsis*
46	qPCR-AtActin2-F	GCACCCTGTTCTTCTTACCG	Forward primer to quantify expression levels of endogenous control reference gene *AtActin2* in *Arabidopsis*
46	qPCR-AtActin2-R	AACCCTCGTAGATTGGCACA	Reverse primer to quantify expression levels of endogenous control reference gene *AtActin2* in *Arabidopsis*
46	LsGGP1-qPCR-F	GATTCCAGGAGATTACGGATTTGTTG	Forward primer to quantify expression levels of target gene *LsGGP1* in lettuce
46	LsGGP1-qPCR-R	CTTCACTTGATTCAAACTGGAAGAGG	Reverse primer to quantify expression levels of target gene *LsGGP1* in lettuce
46	qPCR-LsTIP41-F	TTTGTATGGAGATGAATTGGCTGATA	Forward primer to quantify expression levels of endogenous control reference gene *LsTIP41* in lettuce
46	qPCR-LsTIP41-R	CGTAAGAGAAGAAACCAACAGCTAGG	Reverse primer to quantify expression levels of endogenous control reference gene *LsTIP41* in lettuce

- $CuSO_4$ (Sigma-Aldrich, cat. no. 451657)
- PEG 4000 (Sigma-Aldrich, cat. no. 81240)
- Passive lysis buffer (PLB; 5× ; Promega, cat. no. E1941)
- Bromophenol blue (Abcam, cat. no. 115-39-9)
- Glycine (Thermo Fisher Scientific, cat. no. 15527013)
- Methanol (Sigma-Aldrich, cat. no. 34860) **! CAUTION** Methanol is toxic and should be used under a fume hood. Gloves and lab coat should be worn.
- Concentrated HCl
- Ethanol anhydrous (Macklin, cat. no. E809056)
- Bleach (Huawang, cat. no. YC-XH01011)

Protoplast isolation and transformation

- Sorbitol (Sigma-Aldrich, cat. no. 240850)
- $CaCl_2{\cdot}2H_2O$ (Sigma-Aldrich, cat. no. C7902)
- Sucrose (Sigma-Aldrich, cat. no. V900116)
- 2-(*N*-morpholino)ethanesulfonic acid (MES; Sigma-Aldrich, cat. no. M8250)
- KCl (Sigma-Aldrich, cat. no. P3911)
- Cellulase R-10 (Yakult Pharmaceutical Industry)
- Macerozyme R-10 (Yakult Pharmaceutical Industry)
- Glucose (ApexBio, cat. no. M1210)
- NaCl (Sigma-Aldrich, cat. no. S3014)
- Mannitol (Amresco, cat. no. 0122)

- $MgCl_2$ (Sigma-Aldrich, cat. no. M9272)
- BSA (Sigma-Aldrich, cat. no. A-6793)

Measurement of LUC/REN activities and mRNA levels

- Dual-Luciferase Reporter Assay System (Promega, cat. no. E1910)
- TRIzol (Invitrogen, cat. no. 15596026) **! CAUTION** TRIzol is toxic and should be used under a fume hood. Gloves and lab coat should be worn.
- Chloroform (Wokai, cat. no. 40064966) **! CAUTION** Chloroform is toxic and should be used under a fume hood. Gloves and lab coat should be worn.
- Isopropanol (Macklin, cat. no. I811925) **! CAUTION** Isopropanol is toxic and should be used under a fume hood. Gloves and lab coat should be worn.
- RQ1 RNase-free DNase (Promega, cat. no. M6101)
- RQ1 DNase stop solution (Promega, cat. no. M199A)
- dNTPs (10 mM each; BioDee, cat. no. DE0278)
- RNasin ribonuclease inhibitor (Promega, cat. no. N2518)
- M-MLV reverse transcriptase (Promega, cat. no. M1705)
- $Oligo(dT)_{18}$ (Fermentas/Thermo Fisher Scientific, cat. no. SO132)
- SsoFast EvaGreen Supermix (Bio-Rad, cat. no. 1725201)
- RNase-free water (Amresco, cat. no. E476)

***Agrobacterium*-mediated transformation of *Arabidopsis* and lettuce**

- Murashige & Skoog medium (MS; Duchefa Biochemie B.V., cat. no. M0221.0050)
- Silwet L-77 (Yeasen, cat. no. 41008ES10) **! CAUTION** Silwet L-77 is toxic. Gloves and lab coat should be worn.
- 6-Benzylaminopurine (6-BA; Sigma-Aldrich, cat. no. B3408)
- 1-Naphthaleneacetic acid (NAA; Sigma-Aldrich, cat. no. N0640)
- Hygromycin (Roche, cat. no. 10843555001)
- Kanamycin (50 mg/ml, filter-sterilized; Genview, cat. no. AK177)
- Carbenicillin disodium salt (200 mg/ml, filter-sterilized; Sigma-Aldrich, cat. no. C1389)
- 3-Indole acetic acid (IAA; Abcam, cat. no. ab146402)
- Acetosyringone (Sigma-Aldrich, cat. no. D134406)
- Rifampicin (Duchefa Biochemie, cat. no. R0146) **! CAUTION** Rifampicin is harmful and should be used under a fume hood. Gloves and lab coat should be worn.
- Phytagel (plant tissue agar; Sigma-Aldrich, cat. no. P8169-5Kg)

Identification and screening of transgene-free *uorf* mutants

- DNAquick Plant System (Tiangen Biotech, cat. no. DP321-03)
- FastPfu DNA Polymerase (TransGen Biotech, cat. no. AP221-03)
- High Pure dNTPs (2.5 mM each; TransGen Biotech, cat. no. AD101-12)
- pEASY-Blunt cloning vector (TransGen Biotech, cat. no. CB101-01)

Western blotting analysis

- Anti-BRI1 antibody (raised in the Q. Xie laboratory (http://sourcedb.genetics.cas.cn/zw/zjrck/200907/t20090721_2130985.html) in the Institute of Genetics and Developmental Biology, Chinese Academy of Science, RRID: AB_2801557)
- Anti-PAG (raised in the Q. Xie laboratory (http://sourcedb.genetics.cas.cn/zw/zjrck/200907/t20090721_2130985.html) in the Institute of Genetics and Developmental Biology, Chinese Academy of Science, RRID: AB_2801558)
- Goat anti-rabbit IgG (H+L), HRP conjugate (Proteintech, cat. no. SA00001-2, RRID:AB_2722564)
- Immobilon-NC transfer membranes (Bio-Rad, cat. no. HATF00010)
- Nonfat dry milk powder (Frema, cat. no. 0201V07)
- Chromatography paper (Whatman, cat. no.3030-704)
- DTT (Merck, cat. no. D0632)
- PMSF (Sigma-Aldrich, cat. no. 10837091001)
- Tris base (Amresco, cat. no. 0497)
- Na_2HPO_4 (Sigma-Aldrich, cat. no. 106559)
- KH_2PO_4 (Sigma-Aldrich, cat. no. P9791)
- Ammonium persulfate (APS; Sigma-Aldrich, cat. no. 3678)

- Tween 20 (Amresco, cat. no.0777)
- NP40 (Abcam, cat. no. ab142227)
- Urea (Sigma-Aldrich, cat. no. U0631)
- SDS (Amresco, cat. no. 0227)
- Immobilon Western chemiluminescent HRP substrate (Millipore, cat. no. WBKLS0500)

Treatment of *Arabidopsis* with brassinazole and measurement of foliar ascorbic acid in lettuce

- Brassinazole (Sigma-Aldrich, cat. no. SML1406-5MG)
- EDTA (Sigma-Aldrich, cat. no. E5134)
- Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; Sigma-Aldrich, cat. no. C4706)
- Phosphoric acid (Sigma-Aldrich, cat. no. 79622) **! CAUTION** Phosphoric acid may cause severe skin burns and eye damage; handle it in a fume hood while wearing gloves and a mask.

Evaluation of the off-target effects

- T7EI (ViewSolid Biotech, cat. no. E001L)
- T7EI buffer (10×; ViewSolid Biotech, cat. no. B001)

Equipment

- Incubator–shaker (Eppendorf, model no. Innova42)
- Centrifuge series (Eppendorf, model nos. 5430R, 5810R and 5424)
- Mini-centrifuge (Haimen, model no. LX-300)
- Heating water bath (Memmert, model no. WNB22L4L5)
- Gel Doc XR system (Bio-Rad, model no. 1708195)
- UV–visible light spectrophotometer (Thermo Fisher Scientific, model no. NanoDrop ND-2000)
- Thermoelectric incubator (Memmert, model no. IN160)
- Vacuum pump (Gast, model no. DOA-P504-BN)
- Thermal cycler (C1000 Touch; Bio-Rad, model no. 1851148)
- Petri dish (Greiner Bio-One, cat. no. 633180)
- Syringe sterilization filter (0.45 μm; Sartorius Stedim Biotech, cat. no. 21423103)
- Nylon mesh (40 μm; BD Falcon, cat. no. F613461)
- Single-edge razor blade (Feiying, cat. no. 9875814)
- Falcon tube (50 ml; BD, cat. no. 352070)
- Round-bottom centrifuge tubes (50 ml; Haimeng, cat. no. LXG-50Y)
- Fluorescence microscope (Olympus, model no. SZX16)
- Luminometer (GloMax; Promega, model no. E5311)
- Real-time PCR system (CFX 96 Touch; Bio-Rad, model no. 1855196)
- Mini-Protean Tetra Cell (4-gel system; Bio-Rad, cat. no. 1658004)
- Mini Trans-Blot Cell Module (Bio-Rad, cat. no. 1703811)
- PowerPac HC power supply (Bio-Rad, cat. no. 1645052)
- HPLC system separations module (Waters, model no. e2695)
- Hydrophilic syringe filters (13 mm–0.22 μm; Simsii, cat. no. S13PTFE022L)
- Hemocytometer (Hausser Scientific, cat. no. 3120)

Software

- ImageJ (NIH: http://rsbweb.nih.gov/ij/)
- GraphPad Prism 6.01 (GraphPad Software: https://www.graphpad.com/scientific-software/prism/)
- Cas-OFFinder (http://www.rgenome.net/cas-offinder/)

Reagent setup

Construction of dual-luciferase reporter vectors and sgRNA–Cas9 vectors

Synthesize the sequences of the 35S promoter and 5′ leader, including WT/mutated uORF sequences (synthesized by GenScript), as described in the 'Experimental design' section. See Supplementary Note 1 for the sequences used to generate the data shown in Fig. 4.

LB liquid/solid medium

For liquid medium, dissolve 5 g of yeast extract, 10 g of tryptone and 10 g of NaCl in 800 ml of ddH_2O. Adjust the pH to 7.0 with 1 M NaOH and the volume to 1 liter. Then sterilize by autoclaving.

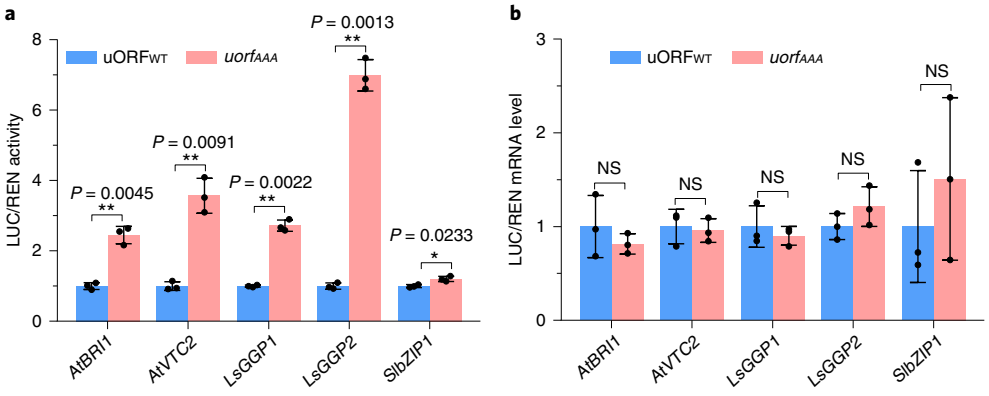

Fig. 4 | The anticipated effects of predicted uORFs on pORFs in transient assays. a,**b**, LUC/REN activities (**a**) and mRNA levels (**b**) in the $uORF_{WT}$ and $uorf_{AAA}$ forms analyzed in dual-luciferase assays of the *AtBRI1*, *AtVTC2*, *LsGGP1*, *LsGGP2* and *SlbZIP1* genes of different species. Translation, rather than transcription, of the pORFs is enhanced, indicating that preventing translation of the five uORFs relieves their inhibitory effect on pORF translation. $uORF_{WT}$ refers to the 5′ leader sequences of the WT uORFs, whereas each $uorf_{AAA}$ has the uORF start codon mutated to AAA. For each gene, the mean LUC/REN activity and mRNA level conferred by $uorf_{AAA}$ were normalized to those of the corresponding $uORF_{WT}$ ($n = 3$). All values are means ± s.d. $^{*}P < 0.05$, $^{**}P < 0.01$; NS, no significant difference by two-tailed Student's *t* test. Adapted from Zhang et al.[23], Springer Nature.

For solid medium, add 14 g of LB agar to the liquid medium and autoclave. Cool the solution to 50–60 °C and add appropriate filter-sterilized antibiotic. Mix well and pour 25 ml of the medium into a sterile Petri dish. The liquid solution and solid medium can be stored at 4 °C for up to 1 month.

MS and 1/2 MS agar plates

Dissolve 4.4 g of MS and 30 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.8 with 1 M KOH and the volume to 1 liter. Add 8 g of plant tissue agar and then sterilize by autoclaving. If necessary, add appropriate filter-sterilized antibiotic after cooling the solution to 50–60 °C and mix well. Pour 25 ml of the medium into a sterile Petri dish. For 1/2 MS agar plates, 2.2 g of MS and 15 g of sucrose are used. Store the MS and 1/2 MS agar plates at 4 °C for up to 1 month.

TVL solution

This solution is 0.3 M sorbitol and 50 mM $CaCl_2$ in ddH_2O. It should be freshly prepared before use.

Enzyme solution for *A. thaliana* protoplasts

This solution is 0.5 M sucrose, 20 mM MES–KOH (pH 5.7), 20 mM $CaCl_2$, 40 mM KCl, 0.4% (wt/vol) cellulase R-10 and 0.4% (wt/vol) macerozyme R-10 in ddH_2O. Incubate the solution at 55 °C for 10 min. Cool to room temperature (22–25 °C) and sterilize the solution with a 0.45-μm filter. This solution should be freshly prepared before use.

W5 solution for *A. thaliana* protoplasts

This solution is 0.1% (wt/vol) glucose, 0.08% (wt/vol) KCl, 0.9% (wt/vol) NaCl, 1.84% (wt/vol) $CaCl_2{\cdot}2H_2O$ and 2 mM MES–KOH (pH 5.7) in ddH_2O. This solution can be stored at room temperature for up to 2 months.

CPW solution for lettuce protoplasts

This solution is 27.2 mg/liter KH_2PO_4, 100 mg/liter KNO_3, 150 mg/liter $CaCl_2$, 250 mg/liter $MgSO_4$, 2.5 mg/liter $Fe_2(SO_4)_3{\cdot}6H_2O$, 0.16 mg/liter KI and 0.00025 mg/liter $CuSO_4$ in ddH_2O. Adjust the pH to 5.8 with 1 M KOH. Store at room temperature for up to 1 month.

Enzyme solution for lettuce protoplasts

This solution is 1.0% (wt/vol) cellulase R-10, 0.5% (wt/vol) macerozyme R-10, 0.45 M mannitol, and 20 mM MES–KOH (pH 5.7) in CPW solution for lettuce protoplasts. Incubate the solution at 55 °C for 10 min. Cool to room temperature and sterilize the solution with a 0.45-μm filter. This solution should be freshly prepared before use.

W5 washing solution for lettuce protoplasts
This solution is 154 mM NaC1, 125 mM $CaC1_2$, 5 mM KC1 and 5 mM glucose in ddH_2O. Adjust the pH to 5.6 with 1 M KOH. This solution can be stored at room temperature for up to 1 month.

CPW 21S
Dissolve 21% (wt/vol) sucrose in CPW solution for lettuce protoplasts and adjust the pH to 5.8 with 1 M KOH. This solution should be freshly prepared before use.

W5 solution for lettuce protoplasts transfection
This solution is 2 mM MES–KOH (pH 5.7), 154 mM NaCl, 125 mM $CaCl_2$ and 5 mM KCl in ddH_2O. This solution can be stored at room temperature for up to 2 months.

WI solution for lettuce protoplasts
This solution is 0.5 M mannitol, 20 mM KCl and 4 mM MES–KOH (pH 5.7) in ddH_2O. This solution can be stored at room temperature for up to 2 months.

Enzyme solution for tomato protoplasts
This solution is 1.5% (wt/vol) cellulase R-10, 0.4% (wt/vol) macerozyme R-10, 20 mM MES–KOH (pH 5.7), 0.4 M mannitol, 20 mM KCl, 10 mM $CaCl_2$ and 0.1% BSA (wt/vol) in ddH_2O. Warm the solution at 55 °C for 10 min. Cool it to room temperature and sterilize the solution with a 0.45-μm filter. This solution should be freshly prepared before use.

W5 solution for tomato protoplasts
This solution is 2 mM MES–KOH (pH 5.7), 125 mM $CaCl_2$, 154 mM NaCl, 0.1 M glucose and 5 mM KCl in ddH_2O. This solution can be stored at room temperature for up to 2 months.

MMG solution
This solution is 4 mM MES–KOH (pH 5.7), 0.4 M mannitol and 15 mM $MgCl_2$ in ddH_2O. This solution should be freshly prepared before use.

PEG solution
Dissolve 40% (wt/vol) PEG 4000 in ddH_2O containing 0.2 M mannitol and 100 mM $CaCl_2$. The PEG solution should be freshly prepared.

1× PLB
Add 1 volume of 5× PLB to 4 volumes of ddH_2O and mix well. This solution should be freshly prepared.

Luciferase assay reagent II
To make Luciferase assay reagent II (LAR II), dissolve a vial of Luciferase Assay Substrate in 10 ml of Luciferase Assay Buffer II (both from the Dual-Luciferase Assay System). Store at −20 °C (≤1 month) or −80 °C (≤1 year).

Stop & Glo reagent
Dilute the 50× Stop & Glo substrate (from the Dual-Luciferase Reporter Assay System) to 1× reagent with Stop & Glo buffer and vortex for 10 s. This solution can be stored at −20 °C for 15 d with no decrease in activity.

Sucrose solution for *Arabidopsis* transformation
Dissolve 50 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.7 with 1 M KOH and the volume to 1 liter. Then add 200 μl of Silwet L-77. This solution should be freshly prepared.

Cocultivation medium for lettuce transformation
Dissolve 4.4 g of MS and 30 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.8 with 1 M KOH and the volume to 1 liter. Add 8 g of agar and then sterilize the solution by autoclaving. Cool the solution to 50–60 °C. Add 0.5 mg of 6-BA and 0.1 mg of NAA. Mix well and pour the medium into sterile Petri dishes. Store the dishes at 4 °C for up to 1 month.

Selection medium for lettuce transformation
Dissolve 4.4 g of MS and 30 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.8 with 1 M KOH and the volume to 1 liter. Add 8 g of agar and then sterilize the solution by autoclaving. Cool the solution to 50–60 °C. Add 0.5 mg of 6-BA, 0.1 mg of NAA, 40 mg of kanamycin and 250 mg of carbenicillin. Mix well and pour the medium into sterile Petri dishes. Store the dishes at 4 °C for up to 1 month.

Shoot outgrowth medium for lettuce transformation
Dissolve 4.4 g of MS and 30 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.8 with 1 M KOH and the volume to 1 liter. Add 8 g of agar and then sterilize the solution by autoclaving. Cool the solution to 50–60 °C. Add 0.046 mg of 6-BA, 0.026 mg of NAA, 40 mg of kanamycin and 250 mg of carbenicillin. Mix well and pour the medium into sterile Petri dishes. Store the dishes at 4 °C for up to 1 month.

Rooting medium for lettuce transformation
Dissolve 2.2 g of MS and 15 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.8 with 1 M KOH and the volume to 1 liter. Add 8 g of agar and then sterilize the solution by autoclaving. Cool the solution to 50–60 °C. Add 0.1 mg of IAA and 250 mg of carbenicillin. Mix well and pour the medium into sterile Petri dishes. Store the dishes at 4 °C for up to 1 month.

Protein extraction buffer
This buffer is 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% NP40 (vol/vol) and 4 M urea. Store at room temperature for up to 2 months. Add PMSF to a final concentration of 1 mM before use.

5× Protein sample buffer
Dissolve 25 mg of bromophenol blue and 0.5 g of SDS in 1.25 ml of 1 M Tris–HCl (pH 6.8) with 2.5 ml of glycerol, and add ddH_2O until the volume is 5 ml. Store at room temperature for up to 2 months.

10× SDS running buffer
Dissolve 30 g of Tris base, 144 g of glycine, and 10 g of SDS in 1 liter of ddH_2O to make 10× SDS running buffer. Immediately before use, dilute to 1× SDS running buffer with ddH_2O. Store at room temperature for up to 2 months.

10× Lamini buffer
Dissolve 151.5 g of Tris base and 720 g of glycine in 1 liter of ddH_2O. Store at room temperature for up to 2 months.

Transfer buffer
Mix 100 ml of 10× Lamini buffer and 200 ml of methanol with 700 ml of ddH_2O. It should be freshly prepared before use.

10× PBS (pH 7.4)
Dissolve 80 g of NaCl, 2 g of KCl, 14.2 g of Na_2HPO_4 and 2.5 g of KH_2PO_4 in ddH_2O to a final volume of 1 liter. Store at room temperature for up to 2 months.

1 M Tris–HCl (pH 6.8)
Dissolve 181.65 g of Tris base in 700 ml of ddH_2O. Adjust the pH to 6.8 with concentrated HCl. Add H_2O to bring the volume to 1 liter. Store at room temperature for up to 2 months.

Extraction buffer for foliar ascorbic acid
Dissolve 4.45 mg of EDTA, 286.65 mg of TCEP and 5 ml of phosphoric acid in 100 ml of ddH_2O. Filter through a 0.45-μm filter and dispense the solution into aliquots. Store at −80 °C for up to 2 months.

Procedure

Analysis and prediction of uORFs ● Timing ~1 d

1 Confirm the DNA sequence of the target gene and locate its 5′ leader sequence in the plant genome database Phytozome v.12.1 (https://phytozome.jgi.doe.gov/pz/portal.html) or NCBI. Predict uORFs using the criteria mentioned above (Experimental design) and then use the sequence alignment software to estimate whether the uORFs are CPuORFs or not on the basis of DNA or amino acid sequence alignment with other species or gene families.

Construction of dual-luciferase reporter vectors for the transient system ● Timing 10–15 d

2 Use a commercial company to synthesize the sequences of the 35S promoter and 5′ leader sequences, including WT/mutated uORF sequences. Different enzyme digestion sites should be individually added at the 5′ and 3′ ends of the synthesized sequences. We use the HindIII restriction enzyme site at 5′ ends and the NcoI restriction enzyme site at 3′ ends.

▲ **CRITICAL STEP** Because the enzyme digestion sites need to be unique, you should check that the chosen sites are the only ones present in the vector and synthesized sequences.

3 Generally, pUC57 vectors containing the synthesized sequences can be obtained from a commercial company. Digest the pUC57 plasmids and the backbone vector pGreenII0800-LUC with HindIII and NcoI in the following reaction and incubate at 37 °C for 1 h.

Component	Volume (μl)	Final concentration
FastDigest Green Buffer, 10×	2	1×
HindIII, 10 U/μl	1	0.5 U/μl
NcoI, 10 U/μl	1	0.5 U/μl
Plasmid DNA	Variable	1 μg
ddH_2O	To 20	

▲ **CRITICAL STEP** Make sure that both chosen restriction enzymes work in this buffer.

4 Run the digestion product on a 2.0% (wt/vol) agarose gel in TAE buffer at 120 V for 20 min. For pUC57, the expected fragment size is the length of the 35S promoter sequence plus the 5′ leader sequence including WT or mutated uORF. For pGreenII0800-LUC, the expected fragment is ~6.3 kb.

5 Cut out the bands containing the expected fragments and purify them with an AxyPrep DNA Gel Extraction Kit, following the manufacturer's instructions; then measure their concentrations with a NanoDrop spectrophotometer.

▲ **CRITICAL STEP** For each plasmid, two or more digestion reactions can be carried out at the same time and combined in a single tube to increase the concentration of the expected fragment.

■ **PAUSE POINT** The purified product can be stored at −20 °C for at least 2 months.

6 Ligate the purified products of the synthesized sequences from Step 5 to the digested pGreenII0800-LUC backbone vector from Step 5. Set up the reaction below and incubate at 22 °C for 1 h:

Component	Volume (μl)	Final concentration
T4 ligation buffer, 10× (from T4 DNA ligase kit)	1	1×
Digested vector	Variable	50 ng
Synthesized fragment	Variable	150–300 ng
T4 ligase, 5 U/μl	0.5	0.25 U/μl
ddH_2O	To 10	

7 Transform the resulting product into competent *E. coli* cells as described in the manufacturer's instructions and incubate them overnight at 37 °C on LB plates with kanamycin.

8 Screen six to eight positive clones by colony PCR using the following reaction with primers for pGreenII0800-LUC-F/R (Table 1):

Component	Volume (μl)	Final concentration
Single colony of bacteria	-	-
2× Taq MasterMix	10	1×
pGreenII0800-LUC-F (10 μM)	1	0.5 μM
pGreenII0800-LUC-R (10 μM)	1	0.5 μM
ddH_2O	To 20	

9 Perform the PCR using the program below; 30 amplification cycles are sufficient:

Cycle no.	Denaturation	Annealing	Extension
1	94 °C, 3 min		
2–31	94 °C, 30 s	60 °C, 30 s	72 °C, 30 s
32			72 °C, 2 min

10 Run the digestion product on a 1.2% (wt/vol) agarose gel in TAE buffer at 120 V for 15 min. A single band is expected. Select two or three positive clones and extract plasmid DNA with a Plasmid Miniprep Kit, following the manufacturer's instructions.
? TROUBLESHOOTING

11 Sequence the plasmids with primers for pGreenII0800-LUC-F2 (Table 1) to identify correct clones, including the synthesized sequences at the designed position of pGreenII0800-LUC.

12 Put the correct clones from Step 11 into 100 ml of LB liquid medium containing 50 μg/ml kanamycin and incubate at 37 °C with shaking at 200 r.p.m. for 14–16 h. Extract the plasmid DNA with a Wizard Plus Midipreps DNA Purification System, following the manufacturer's instructions. The concentration of the plasmid should be at least 1 μg/μl as measured by NanoDrop spectrophotometer.
▲CRITICAL STEP Although having the target sequences synthesized commercially is an easy and reliable way to construct the dual-luciferase reporter vectors, it is costly and time consuming. Therefore, we also recommend the Gibson assembly method for constructing the vectors, which can save time and reduce costs[52].
■PAUSE POINT The plasmid DNA can be stored at −20 °C for several months.

Isolation of protoplasts

13 Isolate protoplasts using option A for *A. thaliana* or option B for lettuce or tomato.

(A) **Isolation of *A. thaliana* protoplasts** ● **Timing ~2 d**

▲CRITICAL Isolate protoplasts from *A. thaliana* seedlings using a previously published protocol with some changes[53].

(i) Use 14-d-old *Arabidopsis* seedlings grown on 1/2 MS medium. Weigh 1–2 g of fresh leaves, put them into a Petri dish with 15 ml of TVL solution (Reagent setup) and chop them up with a new razor blade. Incubate them in the dark for 30 min at room temperature.

(ii) Add 15 ml of filter-sterilized enzyme solution (Reagent setup) and gently rotate the Petri dish to mix well.

(iii) Vacuum-infiltrate the tissues with the enzyme solution for 10 min with a vacuum (~380–508 mmHg). Then gently shake the suspension at 35 r.p.m. for 16 h in the dark at room temperature.

(iv) Swirl the Petri dish gently for 15 s to release the protoplasts; then filter the mixture through a 40-μm nylon mesh to collect the protoplasts in a 50-ml Falcon centrifuge tube. Rinse the tissue in the mesh with 20 ml of W5 solution (Reagent setup) and gently add this to the Falcon tube.
▲CRITICAL STEP Do not shake the final suspension when moving the 50-ml tube before the next step.

(v) Hold the 50-ml centrifuge tube containing protoplasts at 4 °C for 3–4 h.

(vi) Transfer the ring of protoplasts (~10 ml) to a new 50-ml centrifuge tube by pipetting at the interface between the enzyme solution and the W5 solution.

▲ CRITICAL STEP You do not have to be particularly careful not to take any of the upper or lower solution.

▲ CRITICAL STEP You may sometimes fail to see a ring of protoplasts at the interface between the enzyme solution and the W5 solution because of an improper density gradient. If so, pipette all of the protoplast solution into a new 50-ml centrifuge tube without precipitating, and wash with an equal volume of W5 solution in the next step.

(vii) Add 10 ml of W5 solution to wash the protoplasts and mix gently. Centrifuge at 100*g* for 5 min at 25 °C.

(viii) Remove the supernatant by pipetting and resuspend the protoplasts in 10 ml of W5 solution.

(ix) Centrifuge for 5 min at 100*g* at 25 °C and remove as much of the supernatant as possible by pipetting.

(x) Resuspend the protoplasts in 1–3 ml of W5 solution to a concentration of 2.5×10^6 cells/ml, counting them with a hemocytometer under a microscope (100×). Keep the protoplasts on ice for 30 min.

(xi) Remove the W5 supernatant and avoid touching the protoplast pellet. Resuspend the protoplasts at 2.5×10^6 cells/ml in MMG (Reagent setup) solution at room temperature.

▲ CRITICAL STEP Discard the supernatant by pipetting without centrifugation.

(B) **Isolation of lettuce or tomato protoplasts** ● Timing **1–2 d**

(i) Isolate lettuce or tomato protoplasts from seedlings as previously described[21,54].

▲ CRITICAL STEP The concentration of protoplasts should be similar to that in *A. thaliana* after being resuspended in MMG solution.

Transfection and collection of protoplasts ● Timing ~3 d

14 Individually add 20 μg of plasmid DNA (from Step 12) for each vector to 2-ml microcentrifuge tubes. To measure dual-luciferase activity, five independent biological replicates should be done for each vector. For the mRNA analysis, three independent biological replicates should be performed for each vector.

▲ CRITICAL STEP A *GFP* reporter vector is always added as a positive control to estimate the frequency of protoplast transformation by microscope.

▲ CRITICAL STEP For mRNA extraction, double the amount of plasmid DNA. RNase-free tubes should be used.

15 Add 200 μl of protoplasts (5×10^5 cells) to each tube and mix gently.

▲ CRITICAL STEP For mRNA extraction, double the number of protoplasts.

16 Add 220 μl of freshly prepared PEG solution and mix thoroughly by gently tapping the tube.

▲ CRITICAL STEP Shake the PEG solution for at least 1 h to completely dissolve the PEG.

17 Incubate the transfection mixture for 10–15 min at room temperature. For lettuce, the protoplasts should be incubated in the dark. Dilute them with 800 μl of W5 solution and mix well by turning the tube upside down gently to stop transfection.

18 Centrifuge the mixture at 100*g* for 2 min at room temperature, remove the supernatant by pipetting and resuspend the pellet in 1 ml of W5 solution, or for lettuce protoplasts, resuspend the pellet in 1 ml of WI solution.

19 Incubate the protoplasts for 48 h at 23 °C in the dark. After 24 h, check their condition and count the number of GFP-fluorescing cells to assess the transformation frequency using a fluorescence microscope. A frequency of at least 50% is necessary.

? TROUBLESHOOTING

20 Collect the protoplasts 48 h later by centrifuging at 2,000*g* for 10 min at room temperature. Discard as much as possible of the supernatant by pipetting and keep the pellet on ice.

■ PAUSE POINT The protoplast pellet can be frozen in liquid nitrogen and stored at −80 °C for weeks.

Measurement of LUC/REN activity ● Timing 1 d

▲ CRITICAL We recommend that the 1× PLB and 1× Stop & Glo reagent be freshly prepared and the LAR II be dispensed into a 1.5-ml RNase-free centrifuge tube and stored at −80 °C for each experimental use.

21 Turn on the luminometer and select the process 'protocols' → 'DLR-O-INJ'.

22 Add 100 µl of 1× PLB to each sample from Step 20 (~10^6 cells) and vortex for 15–30 s. Incubate the mixtures on ice for 5 min.
▲ CRITICAL STEP Vortex each sample for the same amount of time.
▲ CRITICAL STEP It is not necessary to clear lysates of residual cell debris by centrifugation for this assay.

23 Transfer 20 µl of the lysate to a 1.5-ml microcentrifuge tube with 100 µl of LAR II and use it for the firefly luciferase reporter assay with a luminometer. Mix the reaction by pipetting quickly five times, put it in the luminometer, and measure the firefly luciferase signal.
▲ CRITICAL STEP We do not recommend vortexing the solution in this step.

24 Remove the tube and dispense 100 µl of 1× Stop & Glo reagent into it. Pipette the mixture five times to mix well and measure the *Renilla* luciferase signal.

25 Record firefly luciferase activity (FLU1), *Renilla* luciferase activity (RLU1) and the FLU1/RLU1 ratio. For statistical analyses, exclude the maximum and minimum values of the five replicates. Normalize the data for the experimental samples to the control sample. Use GraphPad Prism 6 or Microsoft Office Excel to calculate statistical significance of differences in LUC/REN activity between the experimental and control groups. Two-tailed, unpaired Student's *t* test should be used to analyze the data. The level of significance can be set at $P < 0.05$, $P < 0.01$, $P < 0.001$, or $P < 0.0001$.
? TROUBLESHOOTING

Quantification of LUC/REN mRNA levels by qRT-PCR ● Timing ~1–2 d

26 Collect all the plant protoplasts (~10^6 cells) for each sample in Step 19 following Step 20 and extract total mRNA from protoplasts pellet using TRIzol. Perform RNA reverse transcription and quantify the mRNA level by qRT-PCR following a previous protocol[55].
▲ CRITICAL STEP Vortex the samples thoroughly after adding TRIzol solution.
▲ CRITICAL STEP Wear a mask and use clean gloves and RNase-free tips.
? TROUBLESHOOTING

27 Calculate the relative expression level of the target gene for each sample according to the following formula:

$$X_{sample} = 2^{-\Delta Ct}, \Delta Ct = Ct_{internal\ gene} - Ct_{target\ gene}$$

where 'Ct' means cycle threshold. Normalize the data for the experimental sample to the control sample. Use GraphPad Prism 6 or Microsoft Office Excel to calculate statistical significance of differences in LUC/REN activity between the experimental and control groups. Two-tailed, unpaired Student's *t* test should be used to analyze the data. The level of significance can be set at $P < 0.05$, $P < 0.01$, $P < 0.001$, or $P < 0.0001$.

Design, construction and delivery of sgRNA–Cas9 vectors ● Timing 7–10 d

28 Analyze the sequence context of the start codon of the target uORF and design an sgRNA to disrupt the translation initiation sequence or its adjacent context (see Box 1 for details).

29 Construct the sgRNA–Cas9 vector used for plant transformation according to previous protocols (see Box 1 for details).
▲ CRITICAL STEP It is best to use a transformation vector with a high efficiency of genome editing in the target plant.
▲ CRITICAL STEP Alternatively, test the activity of the sgRNA in protoplasts by following Steps 13–19 for transient expression and Steps 32–38 for detection of *uorf* mutations.

30 Transform *Arabidopsis* and lettuce using previously described methods[23,47] (see Box 1 for details).

Generation and identification of *uorf* mutants ● Timing 7–9 weeks

31 For *Arabidopsis*, regenerated T_1 seeds can be obtained within 5–8 weeks after transformation in Step 30. Harvest T_1 seeds and germinate the harvested seeds on 1/2 MS agar plates containing 25 µg/ml hygromycin. Distinguish transgenic plantlets from non-transgenic plants by choosing healthy plantlets with green cotyledons and true leaves and developed roots that penetrate the medium used for detection. For lettuce, regenerated T_0 plantlets can be obtained within 5–7 weeks after transformation in Step 30.
▲ CRITICAL STEP Plants transformed with a CRISPR–Cas9 vector without an sgRNA can be used as controls.

Box 1 | Design, construction and delivery of sgRNA–Cas9 vectors ● Timing 7–10 d

Procedure

1 To design sgRNAs targeting a uORF, select target sequences around the start codon by identifying the 5′-NGG-3′ PAM sequence (template strand targeting) or the 5′-CCN-3′ sequence (non-template strand targeting)[48]. If possible, use sgRNAs whose cleavage site is located in the start codon or immediately next to it. To achieve efficient editing, the sgRNA–Cas9 vectors should express Cas9 and the sgRNAs at sufficient concentrations. Therefore, a toolkit of CRISPR–Cas9 expression systems has been developed to meet the needs of gene editing in different plants. Typically, the sgRNAs are driven by U6 or U3 promoters, whereas Cas9 is expressed from constitutively active promoters such as the CaMV *35S* promoter or the Ubiquitin (*Ubi*) promoter[59]. The combination of expression vectors used depends on the plant species. In our experiments, we used vectors AtU6::sgRNA and *pYAO*::Cas9 to generate *Arabidopsis* mutants[42] (option A) and AtU6::sgRNA and *35S*::Cas9 to generate lettuce mutants[51] (option B). (As noted below, for delivery of the vectors, option A is followed by option C; option B is followed by option D.) The detailed steps for the construction and delivery of the sgRNA–Cas9 expression vectors are as follows.

(A) **Construction of sgRNA–Cas9 vectors for *Arabidopsis***

(i) Synthesize the forward and reverse sgRNA oligos.

(ii) Dilute them to final concentrations of 10 μM and set up the following annealing reaction:

Component	Volume (μl)	Final concentration
Forward sgRNA oligos (10 μM)	20	0.2 μM
Reverse sgRNA oligos (10 μM)	20	0.2 μM
ddH_2O	10	

(iii) Anneal the mixture in a PCR machine, using the following program:

Cycle no.	Condition
1	95 °C, 5 min
2	90–10 °C, −10 °C min^{-1}, 8 min
3	10 °C, hold

(iv) Digest the AtU6-26-sgRNA-SK vector with BsaI in the reaction mixture below and incubate at 37 °C for 1 h:

Component	Volume (μl)	Final concentration
CutSmart Buffer, 10×	2	1×
BsaI, 10 U /μl	1	0.5 U/μl
Plasmid DNA	3	1 μg
ddH_2O	To 20	

(v) Purify the digested product following Steps 4 and 5 of the main Procedure.

(vi) Ligate the annealed oligos (5 μl) from step 1A(iii) of this box to the digested product from step 1A(v) of this box according to Step 6 of the main Procedure.

(vii) Proceed as described in Steps 7–11 of the main Procedure. The primers At-gRNA-F/target-oligo-R are used for screening positive clones by colony PCR and the primer At-gRNA-F is used for Sanger sequencing (Table 1).

(viii) Digest the plasmid AtU6-26-sgRNA-SK-target with NheI and SpeI, following Step 3 of the main Procedure. Purify the product (~0.65 kb) by following Steps 4 and 5 of the main Procedure.

(ix) Digest plasmid pCAMBIA1300-*pYAO*:Cas9 with SpeI, following Step 3 of the main Procedure. Add 0.5 μl of CIAP to the digested product and incubate at 37 °C for 30 min. Proceed as described in Steps 4 and 5 of the main Procedure.
▲ **CRITICAL STEP** Do not forget to add CIAP for dephosphorylation, to prevent vectors from self-connecting when digested with one restriction enzyme.

(x) Ligate the digested plasmids AtU6-26-sgRNA-SK-target from step 1A(viii) of this box and pCAMBIA1300-*pYAO*:Cas9 from step 1A (ix) of this box according to Steps 6–11 of the main Procedure. Identify positive clones by colony PCR using primers 1300-sgRNA-F/R, and use 1300-sgRNA-F for Sanger sequencing (Table 1).

(B) **Construction of sgRNA–Cas9 vectors for lettuce**

(i) Clone the target sgRNA into plasmid pKSE401 as described in steps 1A(i–vii) of this box. Use primer pKSE401-U6-F for Sanger sequencing to identify positive clones (Table 1).

2 For delivery of sgRNA–Cas9 vectors into *Arabidopsis*, use option C; for delivery of sgRNA–Cas9 vectors into lettuce, use option D.

(A) **Delivery of sgRNA–Cas9 vectors into *Arabidopsis***

(i) Transform the target plasmid from step 1A(x) of this box into *A. tumefaciens* strain EHA105 by the freeze–thaw method for transformation of *Arabidopsis*[60].

(ii) Transform *Arabidopsis* by the floral dip method[47].

(B) **Delivery of sgRNA–Cas9 vectors into lettuce**

(i) Transform the correct plasmid from step 1B(i) of this box into *A. tumefaciens* strain EHA105 by the freeze–thaw method for transformation of lettuce[60].

(ii) Transform lettuce by leaf discs[23].

32 Extract genomic DNA from the leaves (20–100 mg) of regenerated seedlings using a DNAquick Plant System kit and following the manufacturer's instructions; dissolve the DNA precipitates in ddH_2O to a final concentration of 100–200 ng/μl.

PAUSE POINT The genomic DNA can be stored at −20 °C for at least 3 months.

33 Amplify the target region with specific primers using the following reaction mixture:

Component	Volume (μl)	Final concentration
Genomic DNA	0.5	2–4 ng/μl
PCR-Forward, 10 μM	0.5	0.2 μM
PCR-Reversed, 10 μM	0.5	0.2 μM
FastPfu buffer (from kit), 5×	5	1×
dNTPs, 2.5 mM	2	0.2 mM
FastPfu polymerase, 2.5 U/μl	0.5	0.05 U/μl
ddH_2O	To 25	

CRITICAL STEP High-fidelity DNA polymerases such as FastPfu polymerase should be used to reduce the error rate in PCR amplification.

34 Carry out the PCR reactions in the following conditions:

Cycle no.	Denaturation	Annealing	Extension
1	95 °C, 2 min		
2–36	95 °C, 20 s	Tm °C, 20 s	72 °C, 15 s
37			72 °C, 5 min

Tm, appropriate temperature for PCR primer pair.

35 Run 3 μl of the PCR product on a 1.2% (wt/vol) agarose gel for 15–20 min in TAE buffer at 120 V to check that the desired fragment has been produced.

36 Sequence the PCR product and identify the mutation types using a sequencing chromatogram.

CRITICAL STEP The sequencing chromatograms obtained can be divided into three types: if the sequencing chromatogram has double traces (overlapping peaks) starting from the target site, the mutation is heterozygous or biallelic; if there are more than two superimposed traces (overlapping peaks), the mutation is chimeric; if there is only a single trace, the mutation is homozygous or WT.

? TROUBLESHOOTING

37 Clone the purified PCR products from all the mutants identified in Step 36 into a pEASY-Blunt cloning vector, using the following reaction mix, and incubate at 22 °C for 10 min:

Component	Volume (μl)	Final concentration
Purified PCR products	3–4	4–6 ng/μl
pEASY-Blunt cloning vector	1	
ddH_2O	To 5	

38 Transform *E. coli* cells according to the manufacturer's instructions as described in Step 7. Screen positive clones by colony PCR with the specific primers in Step 33, following Steps 8–10. Select ten clones and sequence them using the common sequencing primers M13F or M13R. Multiple types of *uorf* mutants can be identified.

? TROUBLESHOOTING

Examination of the effects of the mutated *uorf* on the downstream pORF in protoplasts

Timing 19–25 d

39 Synthesize the sequences of 35S promoter and the 5′ leader sequences, including the mutated *uorfs* created by the CRISPR–Cas9 system (identified in Step 38), and test their influence on *LUC* transcription and translation by following Steps 2–27.

Obtainment of transgene-free and homozygous *uorf* mutants in the offspring

● Timing 11–13 weeks

40 Grow all the mutants identified in Step 36 and harvest the seeds of their progenies.
▲ CRITICAL STEP For *Arabidopsis*, grow the plants under the long-day condition of 16 h light/8 h dark, at 20 °C, for ~2 months to harvest seeds. For lettuce, grow the plants under the long-day condition of 16 h light/8 h dark, at 25 °C, for ~3 months to harvest seeds.

41 Germinate the seeds and extract the genomic DNA of the seedlings by following Step 32.
▲ CRITICAL STEP For *Arabidopsis*, sterilize seeds by first treating them with 70% ethanol (vol/vol) for 1 min and then with 10% bleach (vol/vol) for 10 min. Rinse the seeds with sterile water and geminate them on 1/2 MS agar plates. Keep them in darkness at 4 °C for 2 d and germinate at conditions of 16 h light/8 h dark, at 25 °C, for 1–2 weeks. For lettuce, rinse the seeds three times with ddH_2O and put them at room temperature for 4–5 h. Then place them at 4 °C for 48 h and transfer to wet soil.

42 Confirm the genotypes of the seedlings by following Steps 33–38. Identify transgene-free mutants by PCR, using four pairs of primers covering the transformation vector (Table 1). Design a pair of primers to amplify an internal gene as a control (Table 1). The vector DNA used for transformation and WT DNA are used as positive and negative controls, respectively.
▲ CRITICAL STEP To avoid DNA contamination, use unopened tubes, tips and reagents.
▲ CRITICAL STEP 5 μl (or < 8 μl) of each PCR product should be run on the gel to avoid cross-contamination between lanes.
? TROUBLESHOOTING

Evaluation of off-target effects in transgene-free *uorf* mutants by T7EI assays

● Timing ~2 d

43 Search for candidate off-target sites of the sgRNAs, using the online software Cas-OFFinder (http://www.rgenome.net/cas-offinder) according to the standard PAM 5′-NGG-3′, and design and order primers to test ten candidate off-target sites that have minimal mismatches to each gene.

44 Amplify the mutant DNAs from Step 42 with primers designed for the ten candidate off-target sites, following Steps 33 and 34. Amplify the DNA of homozygous mutants mixed with WT DNA or heterozygous mutant DNA as positive controls, and amplify WT DNA with the designed primers as a negative control.
▲ CRITICAL STEP The amplicons should be unique. If not, redesign the primers.

45 Digest the PCR product with T7EI according to the published protocol[48]. If the digested bands are not observed in the mutant sample as in the negative control, no off-target effects occur.
▲ CRITICAL STEP The homozygous mutation cannot be directly detected by T7EI assay. Mix the DNA of mutants with WT DNA to confirm if homozygous off-target mutation occurs.
? TROUBLESHOOTING

Comparison of the mRNA levels of the target gene in the *uorf* mutants and controls by qRT-PCR

● Timing ~1 d

46 Extract and quantify the mRNA levels of *uorf* mutants from Step 42 and controls by following Steps 26 and 27.
? TROUBLESHOOTING

Comparison of protein levels of target genes in *uorf* mutants and controls by western blotting

● Timing ~2 d

47 Prepare the samples from Step 42 for protein extraction and perform western blot analysis following an online website protocol (https://www.abcam.com/protocols/general-western-blot-protocol).
▲ CRITICAL STEP If no antibody is available, transient assays with the dual-luciferase reporter system (from Step 39) can also reveal if the mutated *uorfs* increase translation of the pORF.

Investigation of the relevant phenotypic effects of *uorf* mutants

▲ CRITICAL Consistency of growth conditions and treatments between the *uorf* mutant plants and controls is important.

48 Measure or observe the differences in relevant traits between the *uorf* mutant plants and controls. For example, we used option A to verify the phenotype of *Arabidopsis* hypocotyl growth in different

uorf mutants of *AtBRI1*[56], and option B to examine variations of foliar ascorbic acid concentration in *uorf* mutants of *LsGGP1*[38].

(A) **Phenotype of *Arabidopsis* hypocotyl growth in different *uorf* mutants of *AtBRI1***

● **Timing ~12 d**

(i) Treat *uorf* mutants and controls from Step 42 with brassinazole, as reported in a previous protocol[57]: place the seeds on 1/2 MS medium including 0, 0.2 or 0.8 μM brassinazole for 8 d and test 32 biologically independent samples for each mutant.

(ii) Measure and analyze the hypocotyl lengths of all the samples using Image J (http://rsbweb.nih.gov/ij/).

▲ **CRITICAL STEP** Further concentrations of brassinazole can also be set up to test the varying level of *AtBRI1* translation.

(B) **Measurement of foliar ascorbic acid concentrations in *uorf* mutants of *LsGGP1***

● **Timing ~19 d**

(i) Grow the lettuce seedlings from Step 42 to 18 d old. Perform three biological replicates from three different plants of each isolate.

(ii) Extract and measure foliar ascorbic acid using previously published protocols[58].

Troubleshooting

Troubleshooting advice can be found in Table 2.

Table 2 | Troubleshooting table

Step	Problem	Possible reason	Solution
10	No positive clones when the vectors are constructed	Incomplete digestion of backbone vectors	Digest 1 μg of plasmid DNA with 1 μl of enzyme; incubate the reaction overnight (<16 h)
19	Low efficiency of protoplast transformation	Poor quality of plasmid DNA	Re-extract the plasmid DNA
		Too few or too many protoplasts in MMG	Adjust the concentration of protoplasts before transformation by counting with a hemocytometer
		Incorrect reagent or pH	Check reagent sources and prepare fresh reagents
25	Inconsistent LUC/REN ratios in biologically independent experiments	Inconsistent treatment of independent samples	Try to be consistent at every step in dealing with the protoplasts when measuring LUC/REN activity
26	No or low yield of protoplast mRNA	Too few protoplasts	Double the amount of plasmid DNA and protoplasts
		Degradation of the mRNA	Operate strictly according to the manufacturer's instructions in an RNase-free environment
36	Weak signal of the sequencing chromatograms	Low concentration of PCR product	Increase the amount of PCR product used for sequencing
38	Few *uorf* mutants	Low activity of designed sgRNAs	Validate the activity of the sgRNAs in protoplasts; generate more transgenic plants
42	Strong or weak gel bands from PCR of WT DNA	Homology of the pair of primers between vector and genome sequence	Design new primer pairs and retest
		Contamination of WT DNA during extraction	Use new microcentrifuge tubes, tips and reagents for extracting and amplifying DNA
45	Dispersed bands after T7EI digestion	PCR product not pure	Purify the PCR product before digestion
46	Higher transcript levels of the target gene in *uorf* mutants than controls	Large deletion around the uORF sequence	Use one sgRNA to create multiple *uorf* mutants with small indels

Timing

Step 1, analysis and prediction of uORFs: ~1 d
Steps 2–12, construction of dual-luciferase reporter vectors for transient expression: 10–15 d
Step 13A, isolation of protoplasts of *A. thaliana*: ~2 d
Step 13B, isolation of lettuce or tomato protoplasts: 1–2 d

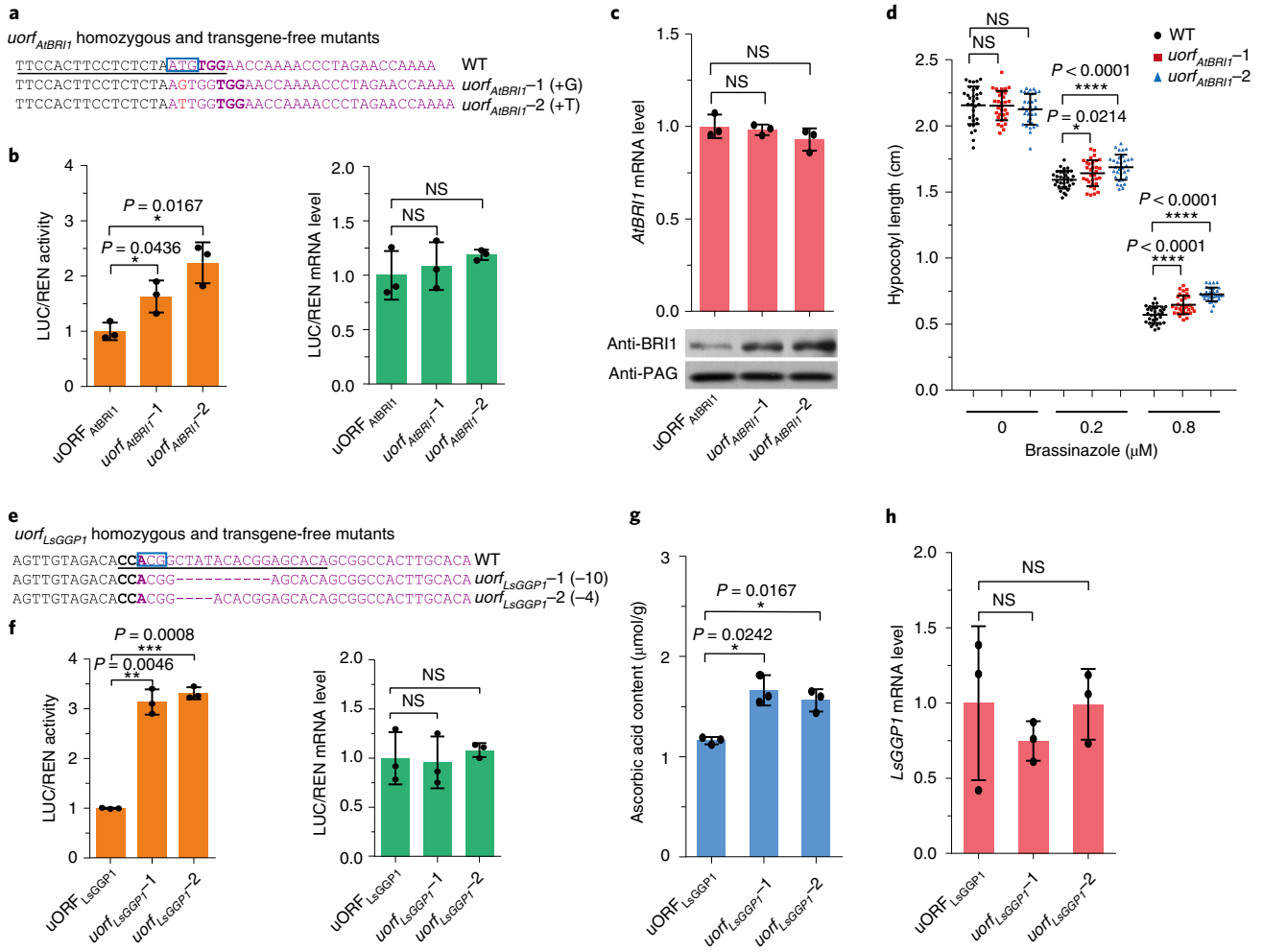

Fig. 5 | Anticipated effects of CRISPR editing on gene translation in *uorf* mutants. a,**e**, Homozygous and transgene-free *uorf* mutants of *AtBRI1* (**a**) and *LsGGP1* (**e**). The uORF sequences are highlighted in purple and the sgRNA target sites with the protospacer-adjacent motifs (bold) are underlined. The inserted nucleotides are labeled in red and the deleted nucleotides are represented by hyphens. The putative start codons of the uORFs are in blue boxes. **b**,**f**, Effects of WT and mutated forms of the uORFs of *AtBRI1* (**b**) and *LsGGP1* (**f**) on LUC/REN activities and mRNA levels in dual-luciferase assays ($n = 3$). The results show that the mutated *uorfs* of *AtBRI1* ($uorf_{AtBRI1}$–1 and $uorf_{AtBRI1}$–2) and *LsGGP1* ($uorf_{LsGGP1}$–1 and $uorf_{LsGGP1}$–2) induced by the CRISPR–Cas9 system stimulated the translation of downstream LUC mRNA relative to the WT uORFs ($uORF_{AtBRI1}$ or $uORF_{LsGGP1}$), whereas their mRNA levels did not differ significantly ($P > 0.05$). **c**, Endogenous *AtBRI1* transcript (top) and protein (bottom) levels showing that the protein products of the transgene-free mutants $uorf_{AtBRI1}$–1 and $uorf_{AtBRI1}$–2 increased without any change in mRNA levels. *Arabidopsis actin2* (At3g18780) was used as internal control for the qRT-PCR assays ($n = 3$), and the 20S proteasome α-subunit G1 (PAG, At2g27020) was used as loading control for the immunoblot assays. **d**, Hypocotyl lengths of $uorf_{AtBRI1}$–1, $uorf_{AtBRI1}$–2 and WT. Overexpression of *AtBRI1* reduced inhibition of *Arabidopsis* hypocotyl growth by brassinazole (0.2 or 0.8 μM) and increased hypocotyl length ($n = 32$). **g**, Increased foliar ascorbic acid contents of the transgene-free mutants $uorf_{LsGGP1}$–1 and $uorf_{LsGGP1}$–2 showing that expression of *LsGGP1* was enhanced. ($n = 3$). **h**, The *LsGGP1* mRNA levels of WT, $uorf_{LsGGP1}$–1 and $uorf_{LsGGP1}$–2 were similar. *LsTIP41* (NM_119592.4) was used as internal control for the qRT-PCR assays ($n = 3$). All values are means ± s.d. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; NS, no significant difference by two-tailed Student's *t* test. Adapted from Zhang et al.[23], Springer Nature.

Steps 14–20, transfection and collection of plant protoplasts: ~3 d
Steps 21–25, measurement of LUC/REN activities: ~1 d
Steps 26 and 27, quantification of LUC/REN mRNA levels by qRT-PCR: 1–2 d
Steps 28–30, design, construction and delivery of sgRNA–Cas9 vectors: 7–10 d
Steps 31–38, generation and identification of *uorf* mutants: 7–9 weeks
Step 39, confirmation of the function of *uorf* mutants in protoplasts: 19–25 d
Steps 40–42, identification of transgene-free and homozygous derivatives: 11–13 weeks
Steps 43–45, analysis of off-target effects in transgene-free *uorf* mutants: ~2 d
Step 46, quantification of mRNA levels of target genes in *uorf* mutants and controls by qRT-PCR: ~1 d
Step 47, comparison of protein levels of target genes in *uorf* mutants and controls by western blotting: ~2 d

Step 48A, exploration of *Arabidopsis* hypocotyl growth in different *uorf* mutants: ~12 d
Step 48B, verification of variation in foliar ascorbic acid concentration in *uorf* mutants of lettuce: ~19 d
Box 1, design, construction and delivery of sgRNA–Cas9 vectors: 7–10 d

Anticipated results

This protocol provides detailed guidelines for manipulating protein translation in plant cells via editing of endogenous uORFs using the CRISPR–Cas9 system. We predicted uORFs of *AtBRI1* and *AtVTC2* in *Arabidopsis*[23], *LsGGP1* and *LsGGP2* in lettuce[23] and *SlbZIP1* in tomato (Supplementary Note 2). In the transient assays, the five uORFs enhanced the translation of downstream *LUC* without affecting mRNA levels when mutations were created at the start codons of the uORFs (Fig. 4a,b). Using CRISPR–Cas9 to disrupt the uORF of *AtBRI1*, we obtained a series of transgene-free and homozygous $uorf_{AtBRI}$ mutants that enabled us to upregulate expression, resulting in different amounts of pORF protein, whereas mRNA levels did not differ significantly ($P > 0.05$) (Fig. 5a–c). Hypocotyl development was consistent with the pORF protein levels (Fig. 5d). Such a strategy was also applicable to *LsGGP1*, and transgene-free $uorf_{LsGGP1}$ mutants with different ascorbic acid contents were obtained (Fig. 5e–h).

In summary, our protocol provides a simple, reliable and generalizable method for enhancing gene translation in plants without introducing exogenous DNA. It can be used to dissect key biological pathways and advance crop breeding. Moreover, owing to the high frequency of uORFs in eukaryotic genes and the rapid development of genome-editing tools (e.g., base editors and Cas9 variants with expanded PAMs), our protocol should be widely applicable.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The authors declare that all data supporting the findings of this study are available in the original paper. Other supporting data are available upon reasonable request to the corresponding author.

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Author contributions

X.S. performed the experiments; Y.W., X.S. and K.C. designed figures; C.G. supervised the project; X.S., H.Z. and C.G. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to C.G.

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Data collection	7500 software v2.0.6 was used to collect the qPCR data.
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Sample size	All the experiments were performed with three biological repeats. And the sample size of hypocotyl growth measurement for each experiment was 32. For Arabidopsis, lettuce and tomato protoplast assay, about 500,000 protoplasts were used for each transfection. The number of mutants were confirmed by Sanger sequencing.
Data exclusions	No data exclusion.
Replication	All attempts at replication were successful. A minimum of 3 biological reps were included.
Randomization	The protoplasts of Arabidopsis, lettuce and tomato were were isolated and randomly separated to each transformation.
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Methods

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☒	☐ ChIP-seq
☒	☐ Flow cytometry
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Antibodies

Antibodies used	The following first and second antibodies were used in this immunoblot assay: rabbit anti-BRI1 (1:1500, from Qi Xie's lab, Institute of Genetics and Developmental Biology, Chinese Academy of Science), rabbit anti-PAG (1:15000, from Qi Xie's lab, Institute of Genetics and Developmental Biology, Chinese Academy of Science) and horseradish peroxidase conjugated goat-anti-rabbit IgG (H+L) (1:2500, SA00001-2, Proteintech).
Validation	These antibodies were validated in previous reports (Cui et al., 2012, Zhang et al., 2015).