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CRISPR-Combo–mediated orthogonal genome editing and transcriptional activation for plant breeding

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CRISPR–Cas nuclease systems, base editors, and CRISPR activation have greatly advanced plant genome engineering. However, the combinatorial approaches for multiplexed orthogonal genome editing and transcriptional regulation were previously unexploited in plants. We have recently established a single Cas9 protein-based CRISPR-Combo platform, enabling efficient multiplexed orthogonal genome editing (double-strand break–mediated genome editing or base editing) and transcriptional activation in plants via engineering the single guide RNA (sgRNA) structure. Here, we provide step-by-step instructions for constructing CRISPR-Combo systems for speed breeding of transgene-free, genome-edited *Arabidopsis* plants and enhancing rice regeneration with more heritable targeted mutations in a hormone-free manner. We also provide guidance on designing efficient sgRNA, *Agrobacterium*-mediated transformation of *Arabidopsis* and rice, rice regeneration without exogenous plant hormones, gene editing evaluation and visual identification of transgene-free *Arabidopsis* plants with high editing activity. With the use of this protocol, it takes ~2 weeks to establish the CRISPR-Combo systems, 4 months to obtain transgene-free genome-edited *Arabidopsis* plants and 4 months to obtain rice plants with enrichment of heritable targeted mutations by hormone-free tissue culture.

Introduction

Since the first demonstration of CRISPR–Cas for programmable DNA cleavage ~10 years ago[1,2], CRISPR–Cas genome-editing tool development and application have experienced explosive growth in the life sciences. In particular, type II CRISPR–Cas9 has emerged as a promising genetic perturbation system, enabling breakthroughs in fundamental and translational research in agriculture[3]. The functional CRISPR–Cas9 complex is composed of a Cas9 nuclease with a dual-guide RNA formed by CRISPR RNAs (crRNAs) hybridized with trans-activating crRNAs[4]. The dual-guide RNA was engineered into a single-guide RNA (sgRNA) by fusing the crRNA and trans-activating crRNA[2]. The protospacer-adjacent motif (PAM) sequence NGG, located immediately 3′ of the protospacer on the DNA strand, is required for the canonical *Streptococcus pyogenes* Cas9–sgRNA-mediated target-site recognition[5]. During target-site recognition, an RNA–DNA heteroduplex between the guide RNA (gRNA) protospacer and the target DNA strand forms[5]. Upon target DNA binding, Cas9 nuclease produces a DNA double-strand break (DSB) via activating its RuvC and HNH nuclease domains, which cleave the PAM-containing non-target DNA strand and gRNA-bound target DNA strand, respectively[6]. The DSB can be repaired by either a nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) pathway. The NHEJ-mediated repair is typically more efficient than HDR and can result in indel (insertion and/or deletion) mutations at the cleavage site that can be harnessed to mediate gene knockout by inducing frameshift mutations and premature stop codons[7]. Cas9-mediated, DSB-dependent, targeted mutagenesis is simple, precise and scalable, empowering scientists to elucidate gene function and dissect the causal linkages between genotype and phenotype even on a genome-wide scale[8–10].

In addition, CRISPR–Cas9-mediated DSB-independent base editing technology has been developed to precisely install targeted point mutations without requiring HDR processes or donor DNA templates[11]. Base editors typically contain a catalytically impaired Cas9 nickase (Cas9n) with an inactivate RuvC nuclease domain and a single-strand DNA deamination enzyme[11]. Currently, two

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main classes of base editors have been developed: cytosine base editors (CBEs)[11] and adenine base editors[12], which catalyze C•G to T•A and A•T to G•C base pair conversion, respectively[13]. Base editors can introduce point mutations at target loci with high precision[14]. Cas9 nuclease also can be converted into a catalytically dead Cas9 (dCas9) by inactivation of both RuvC and HNH catalytic domains, and dCas9 retains competence for RNA-guided DNA binding[15]. Recruitment of transcription activation domains by the dCas9–sgRNA complex to the target gene promoter results in RNA-guided transcriptional activation, known as CRISPRa[16,17], which represents a novel gain-of-function strategy. Compared to the conventional gene-overexpression methods, CRISPRa systems allow for precise, scalable and robust RNA-guided transcription activation[18,19]. The application of CRISPRa systems will enable the dissection of transcription regulatory networks and facilitate metabolic reprogramming[18].

CRISPR–Cas9, base editors, and CRISPRa have been adopted rapidly into basic research and crop improvement[20,21]. However, these tools are typically used separately in practice[22]. Given the fact that each tool has its own capabilities and limitations[22], it is compelling to combine these functionalities to maximize their genome-engineering potential. Just recently, we reported a single Cas9 protein-based CRISPR-Combo platform based on our CRISPRa system, CRISPR-Act3.0 (ref. [19]). CRISPR-Combo has been shown to enable orthogonal genome editing (DSB-mediated genome editing or base editing) and transcriptional activation in the model plant *Arabidopsis thaliana*, food crops *Oryza sativa* (rice) and *Solanum lycopersicum* (tomato) and woody plant *Populus* (poplar)[23]. Beyond the canonical CRISPR systems being capable of either editing or gene regulation, the CRISPR-Combo system allows a user to simultaneously modulate two critical genetic materials, DNA and RNA[23]. Here, we describe a comprehensive protocol for construction of the CRISPR-Combo systems and two applications of CRISPR-Combo to facilitate plant breeding: (i) speed breeding of transgene-free, genome-edited *Arabidopsis* plants and (ii) enhanced rice regeneration and heritable targeted mutations by hormone-free (HF) tissue culture. It is worth noting that this protocol is also applicable to other research objectives and crop species with appropriate modifications.

Development of the protocol

In 2015, Kiani et al. first reported that Cas9 nuclease-dependent and -independent functions can be switched by altering the length of gRNA in human cells[24]. In the native form, Cas9 nuclease is guided by 20-nt protospacers to cleave target sites. By contrast, Cas9 nuclease activity is deprived when truncated sgRNAs with short (e.g., 16-nt) protospacers are used, but Cas9 retains the ability of RNA-guided DNA binding[24]. Fusion of a potent transcriptional activator (VPR) to Cas9 enables simultaneous genome editing and transcriptional activation with 20-nt and 14-nt protospacers, respectively[24]. Subsequently, it was demonstrated that catalytically active CRISPR–Cas12a fused to VPR also enables flexible switching between genome editing and transcriptional activation by altering the sgRNA length in human cells[25].

Motivated by these two previous studies[24,25], we developed a versatile CRISPR-Combo platform based on our previously reported CRISPRa system, CRISPR-Act3.0 (ref. [19]). We established the first sub-system of CRISPR-Combo, Cas9-Act3.0 (ref. [23]), for orthogonal DSB-mediated genome editing and transcriptional activation[23] (Fig. 1a). In the Cas9-Act3.0 system, Cas9 nuclease is co-expressed with a canonical sgRNA scaffold sgRNA1.0 for DSB-mediated genome editing and an engineered sgRNA2.0 that contains two MS2 RNA aptamers, which can recruit transcriptional activators for gene activation (Fig. 1a). Therefore, Cas9-Act3.0 enables transcriptional activation with 15-nt protospacers targeting a promoter region, whereas 20-nt protospacers produce indels[23] (Fig. 1a). On a similar basis, we developed the second sub-system of CRISPR-Combo, CBE-Cas9n-Act3.0 (ref. [23]), which enables orthogonal cytosine base editing (C•G to T•A conversion) and transcriptional activation. In the CBE-Cas9n-Act3.0 system, a highly efficient cytidine deaminase A3A/Y130F and a uracil DNA glycosylase inhibitor (UGI) were fused to the N and C termini of Cas9n, respectively, to generate A3A/Y130F-Cas9n-UGI[26] (Fig. 1b). The A3A/Y130F-Cas9n-UGI was further incorporated into CRISPR-Act3.0 to develop CBE-Cas9n-Act3.0, in which A3A/Y130F-Cas9n-UGI is guided by a 20-nt protospacer within sgRNA1.0 for C-to-T base editing, whereas 15-nt protospacers within sgRNA2.0 lead to gene activation[23] (Fig. 1b).

To demonstrate CRISPR-Combo applications, we focused on overcoming bottlenecks in plant genome editing[27], namely (i) lengthy plant breeding cycle, (ii) difficult and slow plant regeneration and (iii) lack of innovative and efficient selection methods for high-frequency genome-edited plants. In the first demonstration of shortening plant breeding cycle and visual identification of non-transgenic

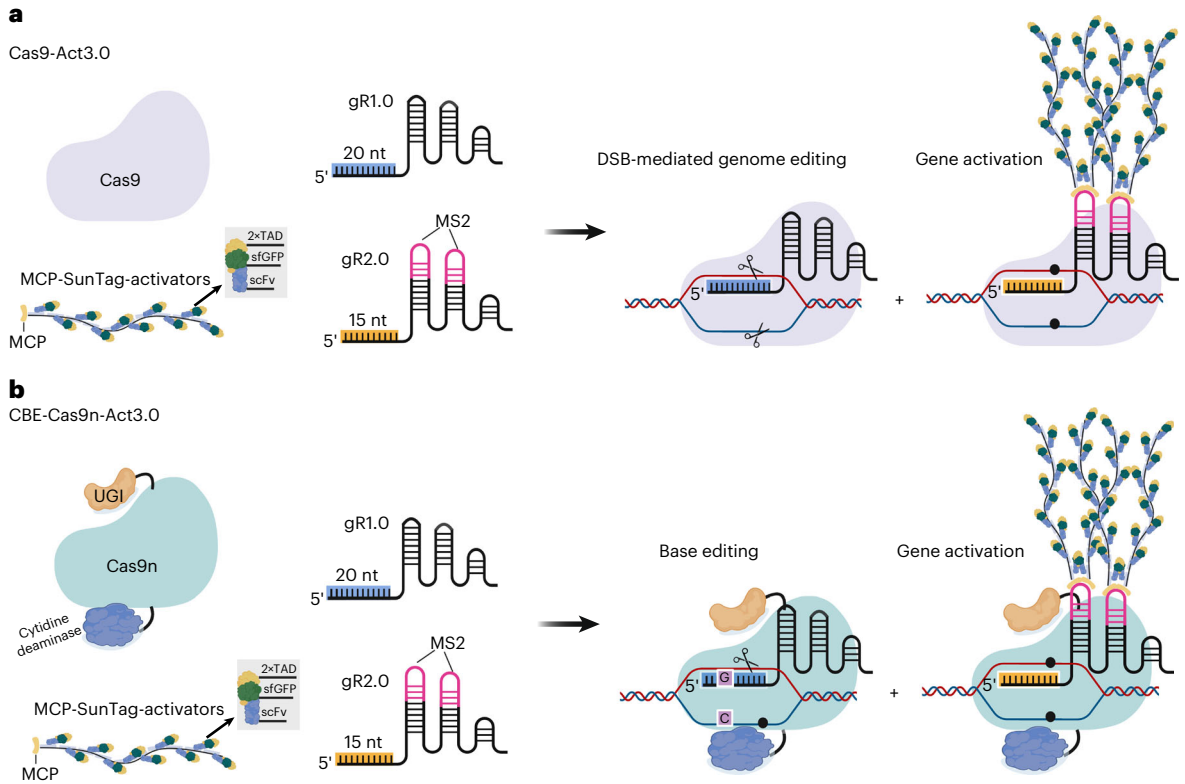

Fig. 1 | Schematic of Cas9-Act3.0 and CBE-Cas9n-Act3.0 CRISPR-Combo systems. a, Diagram of Cas9-Act3.0-mediated orthogonal DSB-mediated genome editing and transcriptional activation. In the Cas9-Act3.0 system, a catalytically active Cas9 nuclease, MS2 bacteriophage coat protein (MCP)-SunTag-activators complex and two different sgRNA scaffolds, gR1.0 and gR2.0, are co-expressed. Each SunTag peptide can recruit 10 copies of activator 2xTAD by scFv fused to sfGFP (scFv–sfGFP). The gR2.0 contains two MS2 RNA aptamers, which enable recruitment of the MCP-SunTag-2xTAD activation complex for activating the target gene by using a 15-nt protospacer and Cas9 nuclease. Simultaneously, the gR1.0 can induce DSB-mediated genome editing with a 20-nt protospacer and Cas9 nuclease. **b**, Diagram of the CBE-Cas9n-Act3.0-mediated orthogonal targeted C-to-T base editing and transcriptional activation. CBE-Cas9n-Act3.0 consists of a Cas9 nickase fused with a cytidine deaminase and uracil glycosylase inhibitor (UGI), an MCP-SunTag-activator complex and gR1.0 and gR2.0. Each SunTag peptide can recruit 10 copies of activator 2xTAD by scFv–sfGFP. The gR2.0 uses two MS2 RNA aptamers to recruit the MCP-SunTag-2xTAD activation complex for activating the target gene by using a 15-nt protospacer. Simultaneously, gR1.0 can induce C-to-T base editing with a 20-nt protospacer and CBE-Cas9n. sfGFP, super-fold green fluorescent protein; scFv, single-chain variable fragment.

genome-edited plants[23], we applied CRISPR-Combo in *Arabidopsis* with activation of the florigen *FT* gene (Fig. 2b). In the second demonstration of boosting plant regeneration and development of novel selection methods[23], we applied CRISPR-Combo in rice by activating the morphogenic gene *OsBBM1* (ref. [28]) (Fig. 2c). We found that this method enables rice plant regeneration from transformed calli without supplying exogenous plant hormones. Importantly, this novel HF plant regeneration method results in transgenic plants with significant enrichment of heritable targeted mutations. These applications are all described in the protocol.

Applications of the method

We have demonstrated the CRISPR-Combo platform for efficient orthogonal genome editing and transcriptional activation in various plants including *Arabidopsis*, rice, tomato and poplar[23]. However, this protocol is not restricted to these specific plant species, and it may be generally applicable to other plants for diverse genetic engineering applications.

With respect to basic research, the CRISPR-Combo platform allows for identification of underlying functional mechanisms of target genes via modulation at both DNA and RNA levels, contributing to our understanding of genetic-interaction networks and biological mechanisms[29]. CRISPR-Combo could perform combinational screens at the whole-genome and transcriptome levels, providing another avenue to high-throughput dissection of the causal relationship between genotype and phenotype and mapping the genetic architecture of complex traits and diseases[30]. In addition, this platform allows researchers to understand the genetic networks of DNA repair modules through the simultaneous introduction of DNA breaks and perturbation of DNA repair-related genes[31].

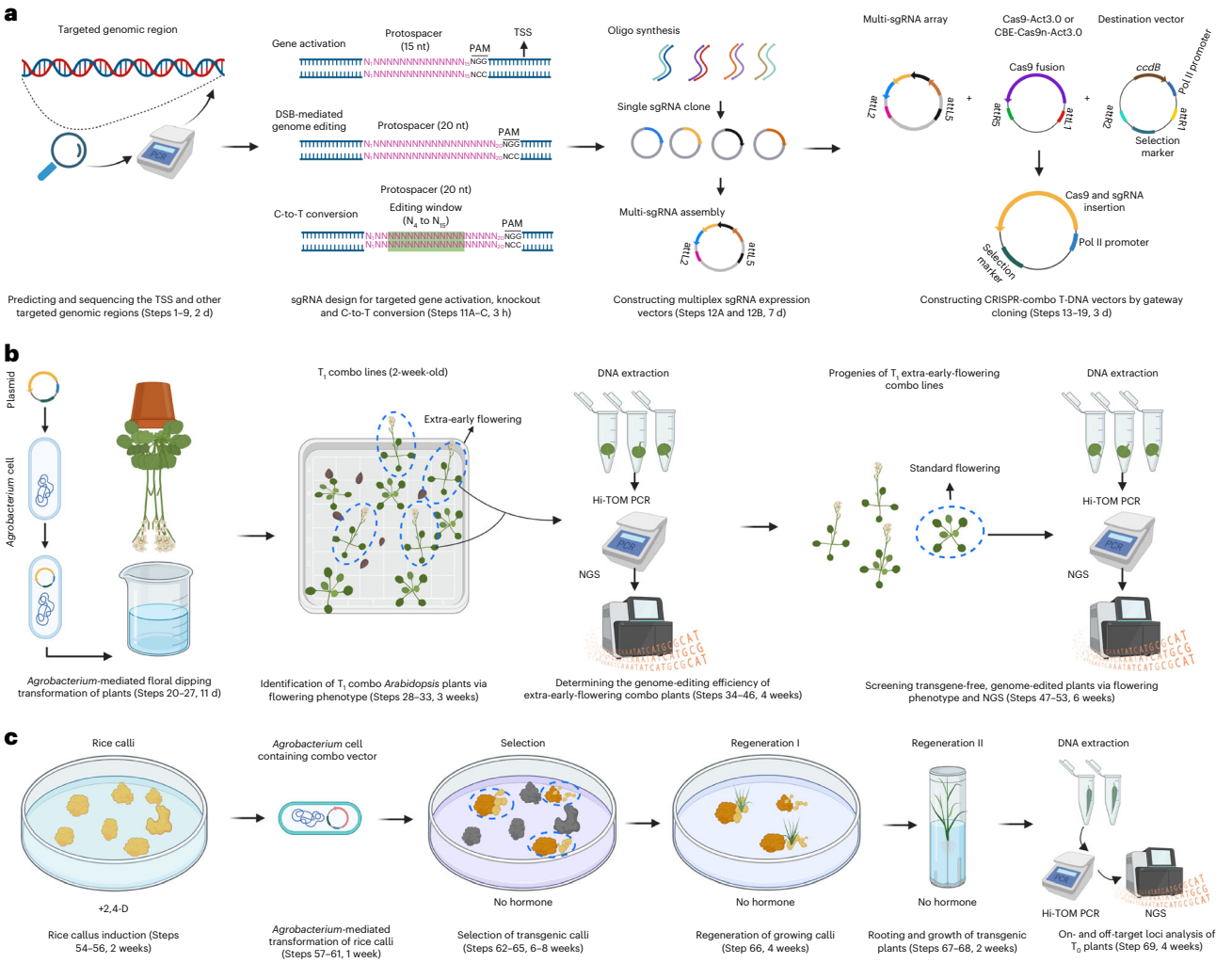

Fig. 2 | Workflow and timeline of the CRISPR-Combo experiments. a, Part 1: construction of CRISPR-Combo vectors. The Combo vector preparation consists of four stages: predicting and confirming the target regions, designing sgRNAs, constructing multi-sgRNA expression vectors and assembling Combo T-DNA vectors. **b**, Part 2: speed breeding of transgene-free, genome-edited *Arabidopsis* plants via Combo-mediated activation of *AtFT*. **c**, Part 3: enhancing rice regeneration and heritable mutations in an HF manner via Combo-mediated activation of *OsBBM1*. Hi-TOM, high-throughput tracking of mutations; TSS, transcription start site; +2,4-D, 2,4-dichlorophenoxyacetic acid.

It could further be used to tailor certain genome-editing outcomes by simultaneously regulating a designated DNA-repair gene or pathway.

The CRISPR-Combo platform undoubtedly holds great promise in plant breeding. This protocol describes the use of CRISPR-Combo to shorten the breeding cycle and aid visual identification of non-transgenic, genome-edited *Arabidopsis* plants by activation of the florigen *FT*[23]. Based on the result that the florigen *FT* was readily activated by CRISPR-Combo in tomatoes[23], we assume that this novel speed-breeding method may be generally applicable in many crops. In addition, this protocol also describes the application of CRISPR-Combo for enhanced rice regeneration and enrichment of heritable mutations in an HF manner by activating the morphogenic gene *OsBBM1* (ref. [23]). Although the Japonica variety Kitaake is used here, this method is fundamentally generic and should be applicable to other rice varieties. Furthermore, users can choose other morphogenic or regulatory genes for activation to overcome the regeneration barrier of very recalcitrant crops. This method also allows users to produce marker-free transgenic plants, because absence of exogenous hormones can be used as the selection condition, reducing public concerns about flow of antibiotic or herbicide selection marker genes into ecosystems. Furthermore, CRISPR-Combo may be used to simultaneously activate morphogenic genes and florigen genes to fast-track the breeding of transgene-free, genome-edited crops and woody plants. CRISPR-Combo also presents an opportunity for metabolic engineering by rewiring metabolic pathways to enhance the production of specific valuable metabolites in plants.

Last, by self-activation of Cas9 and sgRNA expression using sgRNAs targeting Cas9 and sgRNA expression promoters, the CRISPR-Combo system may unleash a positive feedback loop and potentially boost editing and activation efficiency at target loci. In addition, the activator in the platform could be replaced with a repressor or epigenetic modulators, enabling CRISPR-mediated gene interference and epigenome modifications, while simultaneously editing a genomic locus[32]. To expand the targeting scope, other PAM-relaxed or -less Cas9 enzymes such as Cas9-NG[33] and SpRY[23,34] could be applied to this platform.

Comparison with other methods

Recently, single Cas9 protein-based dual base editors such as SPACE[35], A&C-BEmax[36] and Target-ACEmax[37] have enabled the concurrent introduction of C-to-T and A-to-G conversions in human cells. Similar systems such as saturated targeted endogenous mutagenesis editors[38] were demonstrated in plants. Another CRISPR tool, simultaneous and wide editing induced by a single system, has also been developed to generate multiplexed adenine and cytosine base editing as well as NHEJ mutagenesis in plant genomes[39]. These multifunctional genome-editing tools have substantially expanded the diversity of DNA sequence alterations and broadened the capabilities of CRISPR; however, their applications are limited to genome editing.

As aforementioned, CRISPR–Cas9–Cas12a-based combinatorial perturbation systems, Cas9-VPR[24] and Cas12-VPR[25], enable orthogonal DSB-mediated genome editing and transcriptional activation in mammalian cells by altering guide length with a single Cas protein. In both systems, a transcriptional-activator complex (VPR) was directly fused to catalytically active Cas protein, allowing transcriptional activation with truncated 15-nt protospacers, whereas 20-nt protospacers produce indels. However, such designs might result in activation of genome-editing target genes[25]. By contrast, our CRISPR-Combo system Cas9-Act3.0 frees up the Cas9 protein for 'clean' DSB-mediated genome editing and dedicates the sgRNA2.0 scaffold for gene activation, representing a truly orthogonal genome-editing and gene-activation system. Theoretically, it would be complicated and challenging to further fuse other effector domains to the Cas protein within Cas9-VPR[24] and Cas12a-VPR[25] because of the potential functional interference between the new effector and VPR domain. However, the CRISPR-Combo platform retains the capacity to allow for Cas9 fusion with other effector domains such as deaminases. Therefore, a second efficient CRISPR-Combo system, CBE-Cas9n-Act3.0 (ref. [23]), was demonstrated in plants, enabling orthogonal base editing and gene activation. Similar to Cas9-Act3.0, in the CBE-Cas9n-Act3.0, the activation domain will be recruited only by the sgRNA2.0 scaffold, and CBE-Cas9n will be simultaneously used for 'clean' base editing. To the best of our knowledge, CBE-Cas9n-Act3.0 represents the first single Cas9 protein-based CRISPR system for orthogonal base editing and gene activation. Simultaneous gene regulation and nuclease activity had been previously demonstrated by using orthogonal Cas9 proteins[40]; however, these activities were independent from each other. By contrast, in the CRISPR-Combo system, the activation efficiency is positively correlated with the editing activity because both functions are achieved via the same Cas9 protein[23].

Despite considerable progress in recent years, plant regeneration is still the major bottleneck to generating genome-edited plants. Manipulation of exogenous hormones such as auxin and cytokinin in the culture medium could be used to augment plant transformation efficiency. Meanwhile, the co-expression of plant developmental regulators such as *BABY BOOM (BBM)*[41] and *WUSCHEL (WUS)*[42] with CRISPR reagents offers a more straightforward approach to promoting genotype-independent plant regeneration. However, constitutive overexpression of these developmental regulators could result in negative pleiotropic effects, and these transgene cassettes must be excised from genome-edited plants, complicating their applications. More recently, Debernardi et al. demonstrated that the co-expression of GROWTH-REGULATING FACTOR 4 (GRF4) and its cofactor GRF-INTERACTING FACTOR 1 (GIF1) enables substantially higher efficiency of regeneration in wheat, triticale and rice without introducing obvious developmental defects[43]. Even in the absence of exogenous cytokinins, the *GRF4–GIF1* chimera could confer efficient wheat regeneration[43]. By co-delivery with CRISPR reagents, the *GRF4–GIF1* chimera further facilitated the regeneration of genome-edited plants[43]. Using particle bombardment-mediated transient expression of the TaGRF4-TaGIF1 complex, Qiu et al. achieved transgene-free regenerated plants in the first generation[44]. Compared to these aforementioned methods in which plant regeneration and genome-editing events are independent, our CRISPR-Combo approach allows for significant enrichment of targeted germline mutations by promoting regeneration in rice. In addition, neither auxins nor cytokinins are needed for plant

regeneration, paving the way to generate marker-free transgenic plants. Importantly, the regenerated rice plants were fertile and did not show any developmental defects.

Limitations

The main limitations of the CRISPR-Combo platform are related to the caveats associated with the activation and editing efficiency of target genes. First, one limitation is to obtain high activation potency for each target gene. Currently, systematic studies on sgRNA-targeting rules for effective transcriptional activation are still in their infancy[18,45]. Several features identified for target promoters impose stringent requirements on designing efficient sgRNAs for activation[17,19,45–47]. CRISPRa efficiency is highly sensitive to the position of the sgRNA target site relative to the transcription start site (TSS), and effective sgRNAs often target the proximal promoter between −200 bp to +1 TSS[17,19,45–47]. The narrow targeting window ahead of the TSS limits the availability of sgRNA sites with the canonical NGG PAM recognized by Cas9. In addition, because of the natural diversity and complex architecture of promoter sequences, predicting the TSS of promoters is a notoriously difficult task[48]. The precise annotation of TSS is not readily accessible, although many models have been developed to predict the proximal promoters[49–51]. In addition, the chromatin states and basal expression level of target genes can largely constrain the activation potency[17]. Thus, the CRISPRa efficiency is highly dependent on the target gene and its epigenetic state, resulting in activation efficiency varying greatly across genes and species. However, these constraints could be substantially reduced via either pre-screening sgRNA potency by using protoplast systems or using multiple sgRNAs for a single target gene[18,19,52]. In particular, the use of multiple sgRNAs represents a more straightforward strategy to enhance target gene expression. This strategy is also valuable for specific genes that are generally recalcitrant to upregulation at their native loci. To expand the targeting scopes, engineered variants of Cas9 with broadened or altered PAM compatibility can be used, albeit with compromised activation potency[19,23].

Second, as a common feature of all Cas9-mediated genome-editing systems, users should select highly efficient sgRNAs and be aware of the potential off-target effects. Various computational tools are available to predict the efficiency and specificity of sgRNA sites[53]. For example, Cas-OFFinder can help identify all the possible off-target sites for a given sgRNA in a genome[53]. In plants, we recommend applying the online web tool CRISPR-P 2.0 (ref. [54]), which can score all available sgRNAs on the basis of the on-target efficiency and off-target effects. It also provides a comprehensive analysis for gRNA sequences, such as the GC content and secondary structure of sgRNA, which are important for both editing and activation efficiency[19,47,55].

Overview of the procedure

The workflow of this protocol (outlined in Fig. 2) consists of three main parts.

Part 1 consists of construction of the CRISPR-Combo system for orthogonal genome editing and transcriptional activation. Figure 2a shows CRISPR-Combo system selection and TSS prediction (Steps 1–9), target sgRNA design for activation, DSB-mediated genome editing and base editing (Step 11A–C), cloning of multiple sgRNAs into expression constructs (Step 12A–B) and assembly of CRISPR-Combo T-DNA expression vectors (Steps 13–19).

Part 2 consists of speed breeding of transgene-free, genome-edited *Arabidopsis* plants by using CRISPR-Combo. Figure 2b shows delivery of the CRISPR-Combo T-DNA into *Arabidopsis* plants by *Agrobacterium*-mediated transformation (Steps 20–27), identification of T_1 Combo plants with targeted genome editing via flowering phenotype (Steps 28–33), determination of the editing efficiency of extra-early-flowering Combo plants (Steps 34–46) and screening of T_2 transgene-free, genome-edited plants via flowering phenotype and next-generation sequencing (NGS) (Steps 47–53).

Part 3 consists of enhancing rice regeneration and heritable mutations in an HF manner by using CRISPR-Combo. Figure 2c shows callus induction (Steps 54–56), delivery of the CRISPR-Combo T-DNA into rice calli via *Agrobacteria*-mediated transformation (Steps 57–61), selection of transgenic calli (Steps 62–65), regeneration of growing calli (Step 66), rooting and growing of transgenic plants (Steps 67 and 68) and on- and off-target analysis of T_0 Combo plants (Step 69).

Experimental design

CRISPR-Combo system selection

The first step in designing a multiplexed orthogonal genome-editing and transcriptional activation strategy is to select the suitable CRISPR-Combo system. There are two major classes of applications

for which CRISPR-Combo systems are available: Cas9-Act3.0 for orthogonal DSB-mediated genome editing and gene activation and CBE-Cas9n-Act3.0 for orthogonal base editing and gene activation. In both systems, the activation components and architecture are adopted from the CRISPRa system, CRISPR-Act3.0 (ref. [19]). Four copies of MCP (MS2 bacteriophage coat protein)-SunTag-2xTAD (TAL activation domain) transcriptional activation complex can bind the MS2 RNA aptamers within an sgRNA2.0 scaffold for targeted gene activation[19]. In Cas9-Act3.0 (ref. [23]), a *Zea mays* codon-optimized Cas9 (zCas9) from *S. pyogenes* is used, recognizing a canonical NGG PAM. The zCas9 derived from Cas9-Act3.0 can function optimally with 20-nt protospacers for robust DNA cleavage with extremely low off-target editing activity[56,57]. In CBE-Cas9n-Act3.0 (ref. [23]), a potent cytosine deaminase, hA3A-BE3-Y130F (ref. [58]), and a UGI are fused to the N and C termini of zCas9 nickase (Cas9n D10A), respectively. The CBE-Cas9n-Act3.0 has a broad editing window spanning approximately protospacer positions 4–15 in the 5′ to 3′ direction[26]. The substrate nucleotide cytosine within the editing window can be efficiently catalyzed to thymine, enabling the installment of C•G-to-T•A point mutations. Another notable feature of CBE-Cas9n-Act3.0 is that it can introduce premature stop codons (TGA, TAG and TAA) for knocking out protein-coding genes without DSB. In this case, sgRNAs can be designed by using CRISPR-BETS[59]. Users can select a CRISPR-Combo system that best meets the desired genome-editing application.

TSS prediction

There are several website tools publicly available for predicting the TSS and proximal promoter region for the gene of interest[49–51]. In this protocol, we use the genome database Phytozome[60] (https://phytozome-next.jgi.doe.gov/) for retrieving the predicted proximal promoter sequence of the target gene for activation. Phytozome v13 has integrated 274 assembled and annotated plant genomes with comprehensive and uniform annotation. Alternatively, other bioinformatics tools or databases with similar capacities could also be applied in retrieving the proximal promoter sequence. Regardless of which method is used, a proximal promoter of the target gene of ≥300 bp is needed for sgRNA design for targeted gene actviation[17,19,45–47].

Target sgRNA design

Many software tools have been developed to design sgRNAs for targeted mutagenesis[55,61] and base editing[59,62,63]. However, there are no bioinformatics tools available yet to design sgRNAs for CRISPRa in plants. Therefore, in this protocol, we predominantly outline manual methods for sgRNA design for gene activation (Box 1). Notably, the protospacer for both DSB-mediated genome editing and base editing is 20-nt in length, whereas a 15-nt protospacer is used for gene activation. For strong sgRNA transcription, a 5′ guanosine and 5′ adenine is preferred or added in the case of Pol III promoter U6- and U3-driven sgRNA expression, respectively.

Designing sgRNAs for DSB-mediated genome editing

The website tool CRISPR-P v2.0 (ref. [54]) (http://crispr.hzau.edu.cn/CRISPR2/) is recommended for designing sgRNAs with an NGG PAM. CRISPR-P v2.0 currently supports sgRNA design for 49 plant species with the latest genome version and annotation. If the user's genome or sequence is not listed, this web tool allows users to upload custom sequences to design sgRNAs. Usually, multiple sgRNAs are available for one target gene; priority should be given to sgRNAs with high on-targeting efficiency and low off-targeting potential. We recommend sgRNAs targeting early exons for effective creation of frameshifting mutations for knockout.

Designing sgRNAs for base editing

To design sgRNAs for C-to-T base editing by CBE-Cas9n-Act.3.0, the first step is to identify available PAMs enabling the target nucleotide mutations within the editing window (typically, the 4th to 15th base pairs upstream of the PAM). If multiple NGG PAMs are available, the user should give priority to the sgRNAs that have the minimum number of potential off-target sites. The in silico prediction of off-targets can be performed by web tools such as Cas-OFFinder[64] (http://www.rgenome.net/cas-offinder/) and CRISPR-P v2.0 (ref. [54]) (http://crispr.hzau.edu.cn/CRISPR2/). On the contrary, if an NGG PAM cannot be identified that allows for placing the target sequence within the optimal editing window of CBE-Cas9n-Act.3.0, the user can use other engineered Cas9 variants with expanded targeting scopes (e.g., CBE-SpRYn-Act.3.0) (ref. [23]). CBE-SpRYn-Act.3.0 allows for simultaneous PAM-less C-to-T conversions and gene activation; however, its genome-editing[65] and gene-activation

Box 1 | Designing sgRNAs for CRISPR-Combo-mediated gene activation

To design sgRNAs for CRISPR-Combo-mediated gene activation, the first step is to retrieve the proximal promoter sequence ranging from −300 bp to +1 bp from the TSS of the target genes by using the genome database Phytozome or other comparable web tools. Candidate sgRNAs of a 15-nt length are identified by searching for the NGG PAM sequence within the retrieved promoter region. The following principles should be considered when designing efficient sgRNAs for gene activation:

sgRNA position: CRISPRa efficiency is highly sensitive to the position of sgRNA target sites relative to the TSS[16–19,23–25,46,47,54,85,86]. The in silico modeling that allows for prediction of effective CRISPRa target sites is still lacking for plants. Based on the previous studies[17,19,45–47], the proximal promoter region between −200 bp to +1 bp from the TSS is the optimal targeting window for efficient gene activation.

Rule: Design sgRNAs located in the −200 bp to +1 bp window from the TSS, and preferably −180 bp to −30 bp upstream of the TSS[19,23].

GC content of protospacers: Previous studies have identified that the GC content of protospacers contributes toward Cas9 editing activity[87–89]. Protospacers with low or high GC content tend to be less active[88,89]. This feature is also readily applicable for designing efficient sgRNAs for CRISPRa systems[19,47].

Rule: Design protospacers with a GC content ranging from 40% to 65% and with no more than four continuous thymine nucleotides in each protospacer[19].

sgRNA specificity: CRISPR–Cas9, when applied with highly specific sgRNAs in plants, confers very specific genome editing[85,86] and gene activation[47]. Hence, the selection of sgRNAs with high specificity is the most straightforward method to avoid off-target effects[90].

Rule: Select sgRNAs with minimal off-targeting effects by using prediction webtools such as Cas-OFFinder[64] (http://www.rgenome.net/cas-offinder/) and CRISPR-P v2.0 (ref. [54]) (http://crispr.hzau.edu.cn/CRISPR2/).

DNA strand preference: sgRNAs may have DNA strand preference for either editing[89] or transcriptional activation[19].

Rule: Design sgRNAs binding to or complementing the non-coding strand of the proximal promoter may enhance activation efficiency[19].

Other features influencing gene activation efficiency

Chromatin accessibility and state of the target site: CRISPR systems including CRISPRa functionality can be affected by the chromatin accessibility of target sites[91,92]. Open chromatin typically shows high efficiency, and heterochromatin can negatively affect (d)Cas9 proteins' binding and functioning[91–93]. In addition, DNA methylation, in particular CpG methylation, and nucleosome occupancy can affect (d)Cas9 binding by blocking (d)Cas9 access to target DNA[92,94].

Basal transcription level: The currently developed CRISPRa systems generally display an inverse correlation between the basal transcription level and the upregulation level of target genes[17,19,47,52].

Species: Like CRISPR systems[95,96], the CRISPR-Combo platform also has species preference, because different species have varied promoter and codon usage biases and other inherent molecular and physiological characteristics. Currently, the CRISPR-Combo platform has been demonstrated to be efficient in dicots including *A. thaliana*[23], *S. lycopersicum* (tomato)[23], *Populus* (poplar)[23] and monocot rice (*O. sativa*)[23].

Notably, these proposed parameters for designing efficient sgRNAs are also applicable to other CRISPRa systems.

efficiency is relatively low[23]. To install a predefined stop codon, the web tools such as CRISPR-BETS[54] (https://bioinfor.yzu.edu.cn/crisprbets/) and CRISPR-iSTOP[66] (https://www.ciccialab-database.com/istop/#/) can be used.

Designing sgRNAs for activation

For non-plant organisms, the web tools CHOPCHOP v2 (ref. [67]) and CRISPR-ERA[68] have the capability to design sgRNAs for gene activation and repression. However, no such web tools are available for plants[18]. For manual design, the proximal promoter sequence between −300 bp and the +1 TSS of the target genes is first retrieved by using the genome database Phytozome as aforementioned. Then, suitable protospacers with an NGG PAM are identified within the proximal promoter. Considerations for sgRNA design are summarized in Box 1. According to previous reports[17,19,45–47], the −200 bp to +1 bp promoter region represents the optimal targeting window for designing efficient sgRNAs. If multiple NGG PAMs are available within the optimal targeting window, priority should be given to sgRNAs that follow the rules listed in Box 1. Conversely, if no NGG PAM is available within the −200 bp to +1 bp window, sgRNAs targeting upstream of the optimal targeting window can be selected. Among them, certain sgRNAs may work well with CRISPRa systems[19,47,69]. To achieve potent target gene activation, it is highly recommended to pre-screen the sgRNA potency by using transient assays such as protoplast expression systems[70], and the most efficient sgRNA(s) can be identified. Alternatively, without pre-screening, using multiple suitable sgRNAs for a single target gene can have an additive or synergistic effect on programmable gene activation[19,47,52,54].

Assembly of sgRNAs and CRISPR-Combo T-DNA vectors

Once designed, the synthesized protospacer oligonucleotides for either DSB-mediated genome editing or base editing can be cloned into Golden Gate sgRNA modular vectors containing the conventional sgRNA scaffold gRNA1.0 (gR1.0)[19,71] (Fig. 3). By contrast, the protospacer sequences for transcriptional activation need to be cloned into Golden Gate sgRNA modular vectors with an engineered gRNA2.0 (gR2.0) sgRNA scaffold[17,19,23] (Fig. 3), which expresses two MS2 RNA aptamers for recruiting activators. Then, these gR1.0 and gR2.0 expression cassettes can be assembled into an attL5-attL2 sgRNA assembly entry clone in a defined order through Golden Gate Assembly[70] (Fig. 3).

Finally, the attL5-attL2 sgRNA assembly entry clone, the attL1-attR5 Cas-activator entry clone, and the attR1-attR2 destination vector will be simultaneously used for assembly into one CRISPR-Combo T-DNA vector by using MultiSite Gateway cloning technology[70] (Fig. 2a).

Floral dip transformation of *A. thaliana*

Floral dip transformation is used for the transformation of *A. thaliana* with the CRISPR-Combo T-DNA vector for genome editing and activation of the florigen gene *AtFT*[72] (Fig. 2b). First, CRISPR–Act3.0 T-DNA and control plasmids are transformed into preferred *Agrobacterium* competent cells separately according to the freeze-thaw method[73]. Then, the aerial parts or flower buds of 4-week-old *Arabidopsis* plants are dipped in the *Agrobacterium* suspension for 1 min and subsequently incubated in the dark overnight. The treated *Arabidopsis* plants will grow in a growth chamber to harvest T_1 seeds[72].

Identification of the T_1 Combo *Arabidopsis* plants via flowering phenotype

To identify T_1 Combo *Arabidopsis* plants, the collected T_1 seeds are sterilized and plated onto the Murashige and Skoog (MS) medium supplied with related antibiotics (Fig. 2b). The surviving transgenic plants are transferred to the soil to observe the flowering phenotypes, which are defined according to the number of rosette leaves when flower buds appear. On the basis of the flowering phenotypes, these transgenic plants can be classified into extra-early, early and standard flowering groups[23]. Among them, the extra-early- and early-flowering plants represent transgenic lines expressing CRISPR-Combo components (Fig. 2b). In particular, the extra-early-flowering Combo lines not only enable shortening of the plant life cycle from 70 to ~40 d but also confer high editing efficiency[23].

Analysis of editing outcomes of extra-early-flowering Combo lines

NGS technology is recommended to assess genome editing outcomes, including editing efficiency, mutation types and off-target events. Hi-TOM (high-throughput tracking of mutations) that involves two rounds of PCR is used[74]. The first-round PCR primers enable amplification of the region containing the intended target site, and common bridging sequences (5′-ggagtgagtacggtgtgc-3′ and 5′-gagttggatgctggatgg-3′) are added to the 5′ ends (Table 1). PCR amplicons of 150–300 bp with the target sites approximately in the middle are recommended. These amplicons will serve as templates for the second-round PCR[74], using so-called common primers each consisting of a specific adaptor sequence, barcode sequence and bridging sequence (Table 1). The barcode contains two different nucleotides for distinguishing from each other. A total of 12 forward and 8 reverse primers are designed as common primers for the second-round PCR, enabling the barcoding of 96 amplicons in a 96-well format[74] (Table 1). For convenience, these primers can be pre-mixed in a standard 96-well

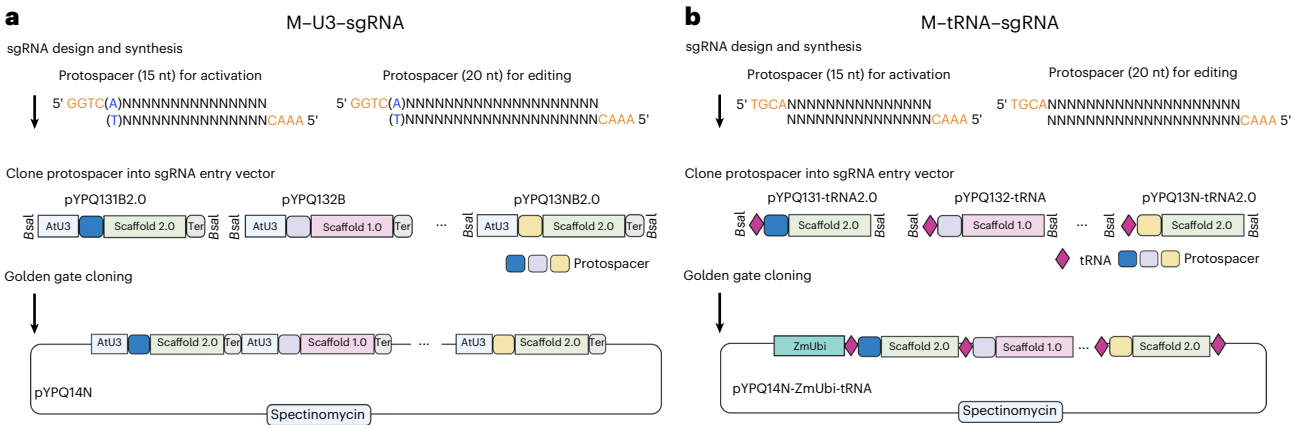

Fig. 3 | Schematic illustrations of assembling multiplexed sgRNA expression vectors. a,**b**, Construction of multiplexed sgRNA expression vectors based on M-U3-sgRNA (**a**) and M-tRNA-sgRNA (**b**) systems. In the M-U3-sgRNA system, each sgRNA is expressed by a Pol III AtU3 promoter. In the M-tRNA-sgRNA system, each sgRNA is flanked by a tRNA, and up to six tRNA-gRNA architectures can be assembled into a single transcript expressed by a Pol II promoter, ZmUbi. In both M-U3-sgRNA and M-tRNA-sgRNA systems, two versions of sgRNA scaffolds, scaffold 1.0 (gR1.0) and scaffold 2.0 (gR2.0), are co-expressed. The gR1.0 represents the canonical sgRNA scaffold for genome editing. The gR2.0 contains two MS2 RNA aptamers enabling the recruitment of activators for targeted gene activation. The nucleotide sequence marked in orange suggests the overhang sequence for sgRNAs. The DNA base 'A' marked in blue indicates that an extra 'A' is added at the 5′ end of the sgRNA, for which its first nucleotide is not 'A', to accommodate U3 promoter-based transcription.

Table 1 | Hi-TOM primers used in this protocol

Step	Name	Purpose	Sequence (5′ to 3′)
35, 53	Hi-TOM-*AtALS*-F	PCR1	**ggagtgagtacggtgtgc**CGATCCTCAGGTAAACCAG
	Hi-TOM-*AtALS*-R	PCR1	**gagttggatgctggatgg**GCTTCCTCAATAATCCTAGGGA
	Hi-TOM-*AtACC2*-F	PCR1	**ggagtgagtacggtgtgc**ACTCAAGGGCGTACAATGA
	Hi-TOM-*AtACC2*-R	PCR1	**gagttggatgctggatgg**CCTTCGAGATCATCTGAGAC
69	Hi-TOM-*OsGW2*-F	PCR1	**ggagtgagtacggtgtgc**CCAGCCACCCAGTATGGAC
	Hi-TOM-*OsGW2*-R	PCR1	**gagttggatgctggatgg**CTTGTTCCGGCCTCTGCA
	Hi-TOM-*OsGN1a*-F	PCR1	**ggagtgagtacggtgtgc**GTGTGGCCACTCGGTGCACG
	Hi-TOM-*OsGN1a*-R	PCR1	**gagttggatgctggatgg**CGCCGACGGTGAGGTGGAGGTA
	Hi-Tom-Off1-*OsGW2*-F	Off-target-PCR1	**ggagtgagtacggtgtgc**CGGATGATTGCATCACAAGGG
	Hi-Tom-Off1-*OsGW2*-R	Off-target-PCR1	**gagttggatgctggatgg**CCAGGGAGAAGCAAGCAGC
	Hi-Tom-Off2-*OsGW2*-F	Off-target-PCR1	**ggagtgagtacggtgtgc**GCCTGTCTACCTCCTTATGGCG
	Hi-Tom-Off2-*OsGW2*-R	Off-target-PCR1	**gagttggatgctggatgg**GCTCCTAGCTCACTATGTGCC
	Hi-Tom-Off3-*OsGW2*-F	Off-target-PCR1	**ggagtgagtacggtgtgc**GATCCCAAGACTGTATGCTGC
	Hi-Tom-Off3-*OsGW2*-R	Off-target-PCR1	**gagttggatgctggatgg**ACGGTCTGAGTCACAGGTAC
	Hi-Tom-Off4-*OsGW2*-F	Off-target-PCR1	**ggagtgagtacggtgtgc**CTTCACGCATGGACGTGTG
	Hi-Tom-Off4-*OsGW2*-R	Off-target-PCR1	**gagttggatgctggatgg**ATCAGCAGTCAGCCACGCA
	Hi-Tom-Off5-*OsGW2*-F	Off-target-PCR1	**ggagtgagtacggtgtgc**TAGCTATAAGAGAACCGCTGGTG
	Hi-Tom-Off5-*OsGW2*-R	Off-target-PCR1	**gagttggatgctggatgg**CTCCTGTTTGCATTTAGGAGCGA
	Hi-Tom-Off1-*OsGN1a*-F	Off-target-PCR1	**ggagtgagtacggtgtgc**ACGAGTCTACGGCTCTACCA
	Hi-Tom-Off1-*OsGN1a*-R	Off-target-PCR1	**gagttggatgctggatgg**GTGGGTTAGCGCACTGGACA
	Hi-Tom-Off2-*OsGN1a*-F	Off-target-PCR1	**ggagtgagtacggtgtgc**CTGCGACGTCGGCACAGC
	Hi-Tom-Off2-*OsGN1a*-R	Off-target-PCR1	**gagttggatgctggatgg**GTCCAACGGGTGCTGAATCTCA
	Hi-Tom-Off3-*OsGN1a*-F	Off-target-PCR1	**ggagtgagtacggtgtgc**GCCGAGCTGGTAGGTGAACTC
	Hi-Tom-Off3-*OsGN1a*-R	Off-target-PCR1	**gagttggatgctggatgg**AGCTCTGAAGCGCCTCTTCT
	Hi-Tom-Off4-*OsGN1a*-F	Off-target-PCR1	**ggagtgagtacggtgtgc**GCTCGTAGGTGAACTCATCGACT
	Hi-Tom-Off4-*OsGN1a*-R	Off-target-PCR1	**gagttggatgctggatgg**CTTGGGCTAGAGGGATCTGCTC
	Hi-Tom-Off5-*OsGN1a*-F	Off-target-PCR1	**ggagtgagtacggtgtgc**GCCGAATCCATGTTTCGACAG
	Hi-Tom-Off5-*OsGN1a*-R	Off-target-PCR1	**gagttggatgctggatgg**CGTTGGTTGCGCTCAACC
35, 53, 69	Hi-TOM-F-1	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttGCGT**tggagtgagtacggtgtgc**
	Hi-TOM-F-2	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttGTAG**tggagtgagtacggtgtgc**
	Hi-TOM-F-3	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttACGC**tggagtgagtacggtgtgc**
	Hi-TOM-F-4	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttCTCG**tggagtgagtacggtgtgc**
	Hi-TOM-F-5	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttGCTC**tggagtgagtacggtgtgc**
	Hi-TOM-F-6	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttAGTC**tggagtgagtacggtgtgc**
	Hi-TOM-F-7	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttCGAC**tggagtgagtacggtgtgc**
	Hi-TOM-F-8	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttGATG**tggagtgagtacggtgtgc**
	Hi-TOM-F-9	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttATAC**tggagtgagtacggtgtgc**
	Hi-TOM-F-10	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttCACA**tggagtgagtacggtgtgc**
	Hi-TOM-F-11	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttGTGC**tggagtgagtacggtgtgc**
	Hi-TOM-F-12	PCR2	*ACACTCTTTCCCTACACGACGCTCTTCCGATCT*gcttACTA**tggagtgagtacggtgtgc**
	Hi-TOM-R-A	PCR2	*GACTGGAGTTCAGACGTGTGCTCTTCCGATCT*ctgtGCGT**tgagttggatgctggatgg**
	Hi-TOM-R-B	PCR2	*GACTGGAGTTCAGACGTGTGCTCTTCCGATCT*ctgtGTAG**tgagttggatgctggatgg**
	Hi-TOM-R-C	PCR2	*GACTGGAGTTCAGACGTGTGCTCTTCCGATCT*ctgtACGC**tgagttggatgctggatgg**
	Hi-TOM-R-D	PCR2	*GACTGGAGTTCAGACGTGTGCTCTTCCGATCT*ctgtCTCG**tgagttggatgctggatgg**
	Hi-TOM-R-E	PCR2	*GACTGGAGTTCAGACGTGTGCTCTTCCGATCT*ctgtGCTC**tgagttggatgctggatgg**
	Hi-TOM-R-F	PCR2	*GACTGGAGTTCAGACGTGTGCTCTTCCGATCT*ctgtAGTC**tgagttggatgctggatgg**
	Hi-TOM-R-G	PCR2	*GACTGGAGTTCAGACGTGTGCTCTTCCGATCT*ctgtCGAC**tgagttggatgctggatgg**
	Hi-TOM-R-H	PCR2	*GACTGGAGTTCAGACGTGTGCTCTTCCGATCT*ctgtGATG**tgagttggatgctggatgg**

The bridging sequences, barcode sequences and adapter sequences are bold, underlined and italic, respectively.

PCR plate. The second-round PCR products with no more than 96 samples can be collected and mixed as one sample for NGS.

NGS-generated data can be analyzed by using various webtools and software packages including CRISPResso2[75] (https://crispresso.pinellolab.partners.org/submission), CRISPR RGEN[63,76] (http://www.rgenome.net/) and CRISPRMatch[77]. We recommend using CRISPRMatch to split the grouped samples into individual sequencing files on the basis of the barcode sequences, and further using either CRISPResso2 or CRISPR RGEN to analyze editing outcomes. Using the Hi-TOM and NGS methods, the editing efficiency and genotype of extra-early-flowering Combo lines are identified and selected for T_2 propagation via selfing to generate transgene-free, genome-edited plants. Alternatively, Sanger sequencing of PCR amplicons can be a fast but low-throughput method to roughly estimate the editing efficiency in each plant. The webtool DSDecode[78] (http://skl.scau.edu.cn/dsdecode/) can be used to decode the Sanger sequencing chromatograms for identification of targeted mutations.

Identification of the T_2 transgene-free, genome-edited *Arabidopsis* plants via the flowering phenotype

The progenies of T_1 extra-early-flowering Combo lines are grown in soil for ~1 month. The T_2 populations also can be classified as extra-early-flowering, early-flowering and standard groups (Fig. 2b). If the ratio of extra-early- and early-flowering plants to the standard plants is ~3:1 (ref. [23]), this indicates that the T_1 parental lines carried only a single transfer DNA (T-DNA) integration event. Only the standard flowering plants are transgene-free candidate plants, because both extra-early-flowering and early-flowering plants contain T-DNA in T_2 populations. Importantly, in the T_2 standard flowering populations, we demonstrated that >90% of plants are genuinely transgene free[23]. This approach significantly narrows down the scope and efforts of screening transgene-free, genome-edited plants because only these standard flowering plants need to be genotyped for genome editing and are free of the transgene (Fig. 2b). Using this method, transgene-free, genome-edited *Arabidopsis* plants can be obtained in ~4 months.

Regeneration of T_0 Combo rice plants in an HF manner

Agrobacterium-mediated transformation of rice calli has been previously described in detail[79]. On the basis of this protocol, we developed a novel method that enables genome-edited rice regeneration in an HF manner via CRISPR-Combo-induced simultaneous activation of the embryogenic trigger gene *OsBBM1* (refs. [28,80]). Briefly, the CRISPR-Combo T-DNA and control plasmids are transformed into the preferred *Agrobacterium* competent cells[73], which are then used to infect 2-week-old rice calli. The transformed calli are cultured on co-cultivation medium without any supplied hormones. The subsequent hygromycin selection and regeneration of transgenic plants is also done in an HF manner[23]. On the basis of this method, transgenic rice plants can be obtained in ~3–4 months with transformation and regeneration frequencies of 35% and 70%, respectively[23].

On- and off-target loci analysis of T_0 Combo rice plants

The candidate off-target loci are predicted by using Cas-OFFinder[64] (http://www.rgenome.net/cas-offinder/) or CRISPR-P v2.0 (ref. [54]) (http://crispr.hzau.edu.cn/CRISPR2/). For each target site, the top five candidate off-target loci are tested. The PCR primers for both on- and off-target analysis are designed according to the aforementioned Hi-TOM method. After two-round Hi-TOM PCR and NGS methods, the genotype and potential off-target events of the regenerated T_0 Combo rice plants are identified.

Controls

To generate transgene-free, genome-edited *Arabidopsis* plants, we use a CRISPR-Combo vector without sgRNA for *AtFT* activation as control. For regenerating the genome-edited rice plants in an HF manner, the CRISPR-Combo vector without the sgRNA for *OsBBM1* activation is used as a control. In short, for all CRISPR-Combo experiments, we recommend inclusion of a CRISPR-Combo control vector without the sgRNA for gene activation. This control allows for the evaluation of the background effects of the CRISPR-Combo T-DNA upon transient and stable transformation. To assess the editing outcomes, activation efficiency or off-target effects, wild-type or transgenic samples containing the CRISPR-Combo vector without sgRNAs can serve as a control.

Materials

Biological materials

- *Arabidopsis thaliana* (ecotype Columbia-0, stock no. CS22681 available at Arabidopsis Biological Resource Center)
- Rice cultivar Kitaake (*O. sativa* ssp *japonica*, stock no. PI 652747 available at US National Plant Germplasm System)
- *Agrobacterium tumefaciens* GV3101 for *Arabidopsis* stable transformation (available at Gold Biotechnology, cat. no. CC-207-5×50)
- *A. tumefaciens* EHA105 for rice stable transformation (available at Gold Biotechnology, cat. no. CC-225-5×50)
- Chemically competent *Escherichia coli* DH5α cells (available at Thermo Fisher Scientific, cat. no. 18265017)

Plasmids

- pYPQ265E2 (Addgene, cat. no. 164719), expressing an A3A/Y130F-CBE for C-to-T base editing
- pYPQ-Cas9-Act3.0 (Addgene, cat. no. 178954), for orthogonal DSB-mediated genome editing and gene activation
- pYPQ-CBE-Cas9n-Act3.0 (Addgene, cat. no. 178955), for orthogonal C-to-T base editing and gene activation
- pYPQ131B2.0 (Addgene, cat. no. 99885) and pYPQ132B2.0 (Addgene, cat. no. 99888), expressing the AtU3-gR2.0 scaffold for targeted gene activation in dicot *Arabidopsis*
- pYPQ133B (Addgene, cat. no. 69283) and pYPQ134B (Addgene, cat. no. 179216), expressing the AtU3-gR1.0 scaffold for targeted gene editing in dicot *Arabidopsis*
- pYPQ131-tRNA2.0 (Addgene, cat. no. 158393) and pYPQ132-tRNA2.0 (Addgene, cat. no. 158394), expressing the tRNA-gR2.0 scaffold without a promoter for targeted gene activation in monocot rice
- pYPQ133-tRNA (Addgene, cat. no. 179212) and pYPQ134-tRNA (Addgene, cat. no. 179213), expressing the tRNA-gR1.0 scaffold without a promoter for targeted gene editing in monocot rice
- pYPQ144 (Addgene, cat. no. 69296), for the assembly of four AtU3-gR2.0 and AtU3-gR1.0 expression cassettes into one vector
- pYPQ144-ZmUbi-tRNA (Addgene, cat. no. 158402), for the assembly of four tRNA-gR2.0 and tRNA-gR1.0 expression cassettes into one sgRNA array driven by a ZmUbi promoter ▲ **CRITICAL** More sgRNA vectors for AtU3-gR1.0/2.0 and tRNA-gR1.0/2.0 expression and their related assembly vectors, such as pYPQ142 to pYPQ146 or pYPQ142-ZmUbi-tRNA to pYPQ146-ZmUbi-tRNA, can be obtained from Addgene. Their catalog numbers are available in the published literature[19,23,70].
- pYPQ202 (Addgene, cat. no. 86198), Gateway-compatible binary T-DNA destination vector for dicot *Arabidopsis* ▲ **CRITICAL** The pYPQ202 harbors a hygromycin resistance (*hpt*) gene driven by the 2X35S cauliflower mosaic virus promoter as the selectable marker. In addition, it provides an *Arabidopsis* ubiquitin promoter AtUBQ10 to express Cas9(n) or the fusion protein of Cas9(n). Users can replace the AtUBQ10 with their promoter of interest by modifying this vector or using a different destination vector.
- pYPQ203 (Addgene, cat. no. 86207), Gateway-compatible binary T-DNA destination vector for monocot rice ▲ **CRITICAL** The pYPQ203 harbors a hygromycin resistance (*hpt*) gene driven by the 2X35S cauliflower mosaic virus promoter as the selectable marker. In addition, it provides a maize ubiquitin promoter ZmUbi to express Cas9(n) or the fusion protein of Cas9(n). Users can replace the ZmUbi with their promoter of interest by modifying this vector or using a different destination vector. ▲ **CRITICAL** The destination vectors pYPQ202 and pYPQ203 each express a toxic *ccdB* gene. To propagate this vector, users need to use *ccdB*-tolerant *E. coli* (such as DB3.1) competent cells. During the Gateway reaction, the *ccdB* gene will be removed.

Reagents

Vector construction

- *Bam*HI-HF (New England Biolabs, cat. no. R3136S; supplied with 10× CutSmart buffer)
- *Esp*3I (*Bsm*BI) (Fisher Scientific, cat. no. FERER0451; supplied with 10× Buffer Tango)
- *Bsa*I-HFv2 (New England Biolabs, cat. no. R3733S; supplied with 10× CutSmart buffer)
- *Eco*RI-HF (New England Biolabs, cat. no. R3101S; supplied with 10× CutSmart buffer)
- *Hind*III-HF (New England Biolabs, cat. no. R3104S; supplied with 10× CutSmart buffer)
- *Xho*I (New England Biolabs, cat. no. R0146S; supplied with 10× CutSmart buffer)
- T4 polynucleotide kinase (PNK) (New England Biolabs, cat. no. M0201S)

- DL-Dithiothreitol (Fisher Scientific, cat. no. FERR0861)
- Instant sticky-end ligase master mix (New England Biolabs, cat. no. M0370L)
- 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal; Fisher Scientific, cat. no. FERR0404)
- IPTG (Fisher Scientific, cat. no. PI34060)
- Gateway LR clonase II enzyme mix (Fisher Scientific, cat. no. 11791020)
- QIAquick gel extraction kit (Qiagen, cat. no. 28706)
- Hi-speed mini plasmid kit (IBI Scientific, cat. no. IB47101)
- DNA ladder (Azura Genomics, cat. no. AZ-1101)
- 6× gel loading dye (New England Biolabs, cat. no. B7024S)
- PR1MA agarose (MIDSCI, cat. no. MIDSCI-500)
- Ethidium bromide (VWR, cat. no. 97064-970) **! CAUTION** Ethidium bromide is a known toxin and mutagen. Gloves and appropriate safety equipment should be worn when handling solutions or stained gels. Solutions and gels should be disposed of according to local regulations.
- Acetic acid glacial (VWR, cat. no. 97064-482)
- Tris base (Fisher Scientific, cat. no. BP152-1)
- EDTA (Fisher Scientific, cat. no. AC118432500)
- Potassium hydroxide (KOH; VWR, cat. no. 18-609-486)
- Glycerol (Fisher Scientific, cat. no. PI17904)
- ddH_2O
- The oligos and primers used in this study are listed in Tables 1 and 2 (Integrated DNA Technologies)

***E. coli* and *A. tumefaciens* cultivation**

- LB broth (Fisher Scientific, cat. no. BP9723-2)
- Bacteriological agar (Fisher Scientific, cat. no. AAJ10906P5)
- Tetracycline hydrochloride (Fisher Scientific, cat. no. BP912-100)
- Kanamycin monosulfate (GoldBio, cat. no. K-120-5)
- Spectinomycin sulfate (Fisher Scientific, cat. no. AAJ6603406)
- Rifampicin (TCI, cat. no. R0079)
- D-Sucrose (Fisher Scientific, cat. no. BP220-1)

Agrobacterium*-mediated transformation of *Arabidopsis

- Silwet L-77 (Fisher Scientific, cat. no. NC1791615) **! CAUTION** Silwet L-77 is toxic. Wear protective gloves and eye protection during operation, and wash thoroughly after handling.
- Bleach (Clorox, https://www.clorox.com/products/clorox-disinfecting-bleach/)
- Tween 20 (Fisher Scientific, cat. no. MP1TWEEN201)
- D-Sucrose (Fisher Scientific, cat. no. BP220-1)
- Murashige and Skoog with vitamins and FeNaEDTA (Caisson Labs, cat. no. MSP20-1LT)
- Hygromycin B (GoldBio, cat. no. H-270-1) **! CAUTION** Hygromycin is a hazardous compound; avoid contact with the eyes, skin or respiratory tract.

***Agrobacterium*-mediated transformation and regeneration of rice**

- Chu's N6 basal medium with vitamins (PhytoTech, cat. no. C167)
- Casamino acids (Fisher Scientific, cat. no. BP1424-100)
- L-Proline (Fisher Scientific, cat. no. AAA1019914)
- D-Sucrose (Fisher Scientific, cat. no. BP220-1)
- Gelrite (Fisher Scientific, cat. no. 50-488-682)
- 2,4-Dichlorophenoxyacetic acid (2,4-D; Fisher Scientific, cat. no. A12467.30)
- Dextrose (D-Glucose) (Fisher Scientific, cat. no. D16-3)
- Acetosyringone (Fisher Scientific, cat. no. 50-255-292)
- Potassium hydroxide (Fisher Scientific, cat. no. AAA1619936)
- Murashige and Skoog with vitamins and FeNaEDTA (Caisson Labs, cat. no. MSP20-1LT)
- D-Sorbitol (Fisher Scientific, cat. no. AA36404A3)
- Myo-inositol (Fisher Scientific, cat. no. AAA1358636)
- Hygromycin B (GoldBio, cat. no. H-270-1)
- Timentin (Fisher Scientific, cat. no. NC9734923)

Table 2 | Other oligos and primers used in this protocol

Step	Name	Purpose	Sequence (5′ to 3′)
12A	M–U3–sgRNA-F	Genome editing (gR1.0)	GGTC(A)N_1NNNNNNNNNNNNNNNNNNN$_{20}$
	M–U3–sgRNA-R	Genome editing (gR1.0)	AAACN$_1$NNNNNNNNNNNNNNNNNNN$_{20}$(T)
	M–U3–sgRNA-F1	Gene activation (gR2.0)	GGTC(A)N_1NNNNNNNNNNNNNNN$_{15}$
	M–U3–sgRNA-R1	Gene activation (gR2.0)	AAACN$_1$NNNNNNNNNNNNNNN$_{15}$(T)
12B	M–tRNA–sgRNA-F	Genome editing (gR1.0)	TGCAN$_1$NNNNNNNNNNNNNNNNNNN$_{20}$
	M–tRNA–sgRNA-R	Genome editing (gR1.0)	AAACN$_1$NNNNNNNNNNNNNNNNNNN$_{20}$
	M–tRNA–sgRNA-F1	Gene activation (gR2.0)	TGCAN$_1$NNNNNNNNNNNNNNN$_{15}$
	M–tRNA–sgRNA-R1	Gene activation (gR2.0)	AAACN$_1$NNNNNNNNNNNNNNN$_{15}$
12A(xi), 12B(ii)	TC14–F2	Sequencing	CAAGCCTGATTGGGAGAAAA
	M13–F1	Sequencing	TTCCCAGTCACGACGTTGTAAAAC
	M13–R1	Sequencing	TTTGAGACACGGGCCAGAGCTGC
12B(ii)	Ubi–intron–F1	Sequencing	CCCTGTTGTTTGGTGTTACTTC
12A(i)	*AtFT*-sgRNA1-F	gRNA oligo	ggtcATGCATTAACTCGGGT
	AtFT-sgRNA1-R	gRNA oligo	aaacACCCGAGTTAATGCAT
	AtFT-sgRNA2-F	gRNA oligo	ggtcATTAGTGTGGTGGGTT
	AtFT-sgRNA2-R	gRNA oligo	aaacAACCCACCACACTAAT
	AtALS-sgRNA-F	gRNA oligo	ggtcACAAGTCCCTCGTCGTATGAT
	AtALS-sgRNA-R	gRNA oligo	aaacATCATACGACGAGGGACTTGT
	AtACC2-sgRNA-F	gRNA oligo	ggtcAGACCAACCGATCATCTTGAC
	AtACC2-sgRNA-R	gRNA oligo	aaacGTCAAGATGATCGGTTGGTCT
12B(i)	*OsBBM1*-sgRNA1-F	gRNA oligo	tgcaGGAAGAGTCTATCTA
	OsBBM1-sgRNA1-R	gRNA oligo	aaacTAGATAGACTCTTCC
	OsBBM1-sgRNA2-F	gRNA oligo	tgcaCCCAATACAAATGCA
	OsBBM1-sgRNA2-R	gRNA oligo	aaacTGCATTTGTATTGGG
	OsGW2-sgRNA-F	gRNA oligo	tgcaGTGTAATCTGGAGAGCTCAC
	OsGW2-sgRNA-R	gRNA oligo	aaacGTGAGCTCTCCAGATTACAC
	OsGN1a-sgRNA-F	gRNA oligo	tgcaTCGCTCGGCCGCCTGCAGGG
	OsGN1a-sgRNA-R	gRNA oligo	aaacCCCTGCAGGCGGCCGAGCGA
52	dzCas9-F	Identification of T-DNA insertion	tggacgcgaccctcatccac
	rbcs-E9t-R	Identification of T-DNA insertion	gttgcctagtgaataagcataatg

DNA extraction and analysis of editing outcomes

- Hexadecyltrimethylammonium bromide (CTAB; Fisher Scientific, cat. no. AC227165000)
- Tris base (Fisher Scientific, cat. no. BP152-1)
- EDTA (Fisher Scientific, cat. no. AC118432500)
- Sodium chloride (Fisher Scientific, cat. no. AC447302500)
- Polyvinylpyrrolidone (Fisher Scientific, cat. no. AAJ6138136)
- Phire tissue direct PCR master mix (Fisher Scientific, cat. no. F170S)
- DNeasy plant pro kit (Qiagen, cat. no. 69204)

Equipment

- Thermal cycler (T100; Bio-Rad, model no. 1861096)
- Centrifuges (Fisher Scientific, model nos. 5425 and 75017012)
- Mini centrifuge (FOUR E'S SCIENTIFIC, no. 4ES0201001)
- Vortex mixer (Scientific Industries, no. 00-SI-0236)
- Dry block heater (Chemglass Life Sciences, model no. BSH1001)
- Incubator set at 37 °C (Fisher Scientific, model no. 650D)
- Shaking incubator set at 37 or 28 °C (New Brunswick, model no. I24R)
- NanoDrop One UV-visible spectrophotometer (Fisher Scientific, cat. no. 13-400-519)
- GelDoc Go gel imaging system (Bio-Rad, model no. 12009077)
- pH meter (Fisher Scientific, model no. 13-636-AE153)
- Gel electrophoresis tanks (Bio-Rad, model nos. 1704466 and 1704508)
- Gel electrophoresis power source (Fisher Scientific, model no. FBEC300XL)
- Laminar flow hood (NuAire, model no. NU-240-430)
- Autoclave (STERIS, model no. AMSCO 250LS)
- Magnetic stirrer (Fisher Scientific, cat. no. SP88857200)

- Magnetic stir bar (VWR, cat. no. 58948-397)
- Vacuum manifold (Qiagen, cat. no. 19413)
- Scale (Mettler Toledo, model no. ML1602T/00)
- Sterile plastic Petri plates, 100 × 15 mm (Fisher Scientific, cat. no. 07-202-010)
- Disposable sterilized syringe, 10 ml (Fisher Scientific, cat. no. 14-817-54)
- Conical sterile centrifuge tubes, 50 ml (Fisher Scientific, cat. no. 12-565-270)
- Conical sterile culture tubes, 15 ml (Fisher Scientific, cat. no. 22-010-078)
- Sterile syringe filter, 0.2 μm (Fisher Scientific, cat. no. 723-2520)
- Filter paper, 9 cm (Fisher Scientific, cat. no. 09-801B)
- Plating beads (Millipore, cat. no. 71013-3)
- 200-μl eight-tube strips (USA Scientific, cat. no. 1402-4780)
- 1.7-ml microcentrifuge tubes (Fisher Scientific, cat. no. 50-196-2463)
- 2.0-ml microcentrifuge tubes (Fisher Scientific, cat. no. S35859)
- Rimless flat-bottom glass tubes (Fisher Scientific, cat. no. 14-957-85C)
- Plant pots (any brand)
- Ultrapure water system (any brand)

Software and webtools

- SnapGene Viewer (Dotmatics, version 4.3.11)
- CRISPR-P v2.0 (http://crispr.hzau.edu.cn/CRISPR2/)
- Phytozome (https://phytozome-next.jgi.doe.gov/)
- CRISPResso2 (https://crispresso.pinellolab.partners.org/submission)
- CRISPR RGEN (http://www.rgenome.net/)
- CRISPRMatch (https://github.com/zhangtaolab/CRISPRMatch)
- TAIR — The *Arabidopsis* Information Resource (https://www.Arabidopsis.org/)
- Rice Genome Annotation Project (http://rice.uga.edu/)

Reagent setup

▲ **CRITICAL** All media were prepared by using ddH_2O.

LB broth/agar medium

Broth medium

Dissolve 25 g of LB broth in 800 ml of ddH_2O. Adjust the pH to 7.2 by using 1 M KOH and adjust the volume to 1 liter with ddH_2O. Sterilize by autoclaving for 30 min.

Agar medium

Add 15 g of agar to the broth medium (pH 7.2). Sterilize by autoclaving for 30 min. Cool the medium to ~60 °C and add related antibiotics. Mix well and pour the agar medium into 40 sterile Petri dishes in a laminar flow hood, solidify and dry. The LB broth and plated agar medium can be stored at 4 °C for 2 months.

1/2 MS for *Arabidopsis* germination

Mix and dissolve 2.2 g of MS salts and vitamins and 10 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.8 by using 1 M KOH and add 8 g of agar. Adjust the final volume to 1 liter with ddH_2O. Sterilize by autoclaving for 30 min. Cool the medium to ~50 °C and add related antibiotics. Mix well and pour the agar medium into 40 sterile Petri dishes in a laminar flow hood, solidify and dry. The plated 1/2 MS medium can be stored at 4 °C for 2 months.

Floral dip solution for *Arabidopsis* transformation

Dissolve 25 g of sucrose in 400 ml of ddH_2O. Adjust the pH to 5.7 by using 1 M KOH and adjust the final volume to 500 ml with ddH_2O. Then, add 100 μl of Silwet L-77 per 500 ml of resuspended solution and mix well with a stirring bar. This solution should be freshly prepared on the day of transformation.

Selection medium for CRISPR-Combo *Arabidopsis* lines

Mix and dissolve 4.3 g of MS salts and vitamins and 10 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.8 by using 1 M KOH and add 8 g of agar. Adjust the final volume to 1 liter with ddH_2O and then sterilize the solution by autoclaving for 30 min. Cool the medium to ~50 °C and add 300 μl of 1,000× hygromycin (50 mg/ml) and 1 ml of 1,000× Timentin (100 mg/ml). Mix well and pour the medium into 40 sterile Petri dishes in a laminar flow hood, solidify and dry. The plated selection

medium can be stored at 4 °C for 1 month. ▲ CRITICAL Select the appropriate antibiotics according to your construct.

Rice callus induction medium
Mix and dissolve 4 g of N6 basal medium with vitamins, 300 mg of casamino acids, 2.8 g of L-proline and 30 g of sucrose in 800 ml of ddH_2O. Adjust to pH 5.8 by using 1 M KOH. Add 4 g of gelrite and adjust the final volume to 1 liter with ddH_2O. Sterilize the solution by autoclaving for 30 min. Cool the medium to ~60 °C and add 1 ml of 1,000× 2,4-D (2 mg/ml) in a laminar flow hood. Mix well and pour the medium into 30 sterile Petri dishes, solidify and dry. The plated selection medium can be stored at 4 °C for 1 month.

Rice infection medium
Mix and dissolve 4 g of N6 basal medium with vitamins, 0.7 g of L-proline and 68.4 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.2 by using 1 M KOH and adjust the final volume to 900 ml. Sterilize the solution by autoclaving for 30 min. This medium can be stored at 4 °C for 1 month. Before use, add 100 ml of filter-sterilized glucose solution (360 g/liter), 0.75 ml of 1,000× 2,4-D (2 mg/ml) and 1 ml of 1,000× acetosyringone (20 mg/ml) in a laminar flow hood. ▲ CRITICAL Acetosyringone is critical for promoting the infection process of *Agrobacterium*.

Rice co-culture medium
Mix and dissolve 4 g of N6 basal medium with vitamins, 300 mg of casamino acids and 30 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.8 by using 1 M KOH. Add 4 g of gelrite and adjust the final volume to 900 ml. Sterilize the solution by autoclaving for 30 min. Cool the medium to ~60 °C and add 100 ml of filter-sterilized glucose solution (100 g/l) and 1 ml of 1,000× acetosyringone (20 mg/ml) in a laminar flow hood. Mix well and pour the medium into 30 sterile Petri dishes, solidify and dry. This plated medium can be stored at 4 °C for 1 month. ▲ CRITICAL Autoclaving glucose together with other nutrient components can enhance its degradation and generate associated toxic products. Thus, it is advisable to sterilize it separately by using a 0.22-μm filter. Importantly, no hormone is supplied in this medium to generate HF Combo rice lines.

Rice selection medium
Mix and dissolve 4 g of N6 basal medium with vitamins, 300 mg of casamino acids, 2.8 g of L-proline and 30 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.8 by using 1 M KOH. Add 4 g of gelrite and adjust the final volume to 1 liter. Sterilize the solution by autoclaving for 30 min. Cool the medium to ~60 °C and add 1 ml of 1,000× hygromycin (50 mg/ml) and 2 ml of 1,000× Timentin (100 mg/ml) in a laminar flow hood. Mix well and pour the medium into 30 sterile Petri dishes, solidify and dry. This plated medium can be stored at 4 °C for 1 month. ▲ CRITICAL No hormone is supplied in this medium to generate HF Combo rice lines.

Rice regeneration medium I
Mix and dissolve 4.43 g of MS salts and vitamins, 2 g of casamino acids, 30 g of sorbitol and 30 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.8 by using 1 M KOH. Add 4 g of gelrite and adjust the final volume to 1 liter. Sterilize the solution by autoclaving for 30 min. Cool the medium to ~60 °C and add 1 ml of 1,000× hygromycin (50 mg/ml) and 2 ml of 1,000× Timentin (100 mg/ml) in a laminar flow hood. Mix well and pour the medium into 30 sterile Petri dishes, solidify and dry. The plated medium can be stored at 4 °C for 1 month. ▲ CRITICAL No hormone is supplied in this medium to generate HF Combo rice lines.

Rice regeneration medium II
Mix and dissolve 4.43 g of MS salts and vitamins, 0.1 g of myo-inositol and 30 g of sucrose in 800 ml of ddH_2O. Adjust the pH to 5.8 by using 1 M KOH. Add 3 g of gelrite and adjust the final volume to 1 liter. Sterilize the solution by autoclaving for 30 min. Cool the medium to ~60 °C and add 2 ml of 1,000× Timentin (100 mg/ml) in a laminar flow hood. Mix well and pour the medium into 35 sterile flat-bottom glass tubes, solidify and dry. The prepared medium can be stored at 4 °C for 1 month.

CTAB extraction buffer
Mix 30 ml of 10% (vol/vol) CTAB stock solution, 28 ml of 5 M NaCl, 4 ml of 0.5 M EDTA (pH 8.0), 10 ml of 1 M Tris-Cl (pH 8.0) and 3 g of polyvinylpyrrolidone. Next, bring the final volume to 100 ml by using ddH_2O. The CTAB extraction buffer can be stored at room temperature (25 °C) for ≥6 months.

50× TAE stock solution
Dissolve 242 g of Tris base in 600 ml of ddH_2O and then add 100 ml of 0.5 M EDTA (pH 8.0) and 57.1 ml of acetic acid. Mix well by using a stir bar and bring the final volume to 1 liter by using ddH_2O. The TAE stock solution can be stored at room temperature for ≥6 months. The 1× TAE working solution is 40 mM Tris-acetate/1 mM EDTA.

0.5 M EDTA (pH 8.0)
Dissolve 186.12 g of EDTA in 800 ml of ddH_2O. Add ~16–18 g of NaOH pellets to adjust the pH to 8.0. EDTA will not dissolve until the pH is near 8.0. Bring the final volume to 1 liter with ddH_2O. The 0.5 M EDTA (pH 8.0) solution can be stored at room temperature for 1 year.

1 M Tris-Cl (pH 8.0)
Dissolve 121.1 g of Tris in ~800 ml of ddH_2O. Bring the pH down to 8.0 by using ~50 ml of concentrated HCl. Bring the final volume to 1 liter with ddH_2O. The 1 M Tris-Cl (pH 8.0) solution can be stored at 4 °C for 2 months.

1,000× hygromycin (50 mg/ml)
Dissolve hygromycin B in ddH_2O at a concentration of 50 mg/ml (wt/vol%) and sterilize by using a 0.22-μm syringe filter. Store at −20 °C for a few months.

1,000× Timentin (100 mg/ml)
Dissolve Timentin in ddH_2O at a concentration of 100 mg/ml (wt/vol%) and sterilize by using a 0.22-μm syringe filter. Store at −20 °C for a few months.

1,000× 2,4-D (2 mg/ml)
Dissolve 2,4-D in ethanol at a concentration of 2 mg/ml (wt/vol%) and sterilize by using a 0.22-μm syringe filter. Store at −20 °C for a few months.

1,000× acetosyringone (20 mg/ml)
Dissolve acetosyringone in DMSO at a concentration of 20 mg/ml (wt/vol%) and sterilize by using a 0.22-μm syringe filter. Store at −20 °C for a few months.

1,000× tetracycline (10 mg/ml)
Dissolve tetracycline in ddH_2O at a concentration of 10 mg/ml (wt/vol%) and sterilize by using a 0.22-μm syringe filter. Store at −20 °C for a few months.

1,000× spectinomycin (50 mg/ml)
Dissolve spectinomycin in ddH_2O at a concentration of 50 mg/ml (wt/vol%) and sterilize by using a 0.22-μm syringe filter. Store at −20 °C for a few months.

1,000× kanamycin (50 mg/ml)
Dissolve kanamycin in ddH_2O at a concentration of 50 mg/ml (wt/vol%) and sterilize by using a 0.22-μm syringe filter. Store at −20 °C for a few months.

1,000× rifampicin (50 mg/ml)
Dissolve rifampicin in methanol at a concentration of 50 mg/ml (wt/vol%) and sterilize by using a 0.22-μm syringe filter. Store at −20 °C for a few months. **! CAUTION** Methanol is extremely poisonous. All operations should be performed in a fume hood; avoid contact with the eyes, skin and respiratory tract.

20 mg/ml X-gal
Dissolve 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside in dimethylformamide at a concentration of 20 mg/ml (wt/vol%) and sterilize by using a 0.22-μm syringe filter. Wrap the tube containing X-gal solution by using aluminum foil to prevent damage from light. Store at −20 °C for a few months.

0.1 M IPTG

Dissolve IPTG in ddH_2O at a concentration of 0.1 M and sterilize by using a 0.22-μm syringe filter. Store at −20 °C for a few months.

Procedure

Part 1: construction of the CRISPR-Combo system for orthogonal genome editing and transcriptional activation

Sequencing of target sites ● **Timing 2 d**

▲ **CRITICAL** It is important to sequence the targeted genomic regions containing the sgRNA sites first, because different varieties may have sequence variations, especially in promoter regions[81]. To ensure successful sgRNA design, the user can use Sanger sequencing to confirm the target sequence (Fig. 2a).

1 The proximal promoter sequence and other genomic regions of the target gene can be retrieved from the plant genome database Phytozome v.13 (https://phytozome-next.jgi.doe.gov/) or the genome database of the target species. To retrieve the proximal promoter sequence by using Phytozome v.13, click on the 'Tools' tab at the top and select 'Biomart'; then, under 'CHOOSE DATASET', select 'V13 Genomes and Families'. Again, under 'CHOOSE DATASET', select 'Phytozome V13 Genomes'. On the left-hand side, go to 'Filters - click to specify', and under 'ORGANISM', click 'Select Organism' and select the target species; under 'GENE', click 'ID List Filter' and then copy the gene ID into the empty column. Again, on the left-hand side, go to 'Attributes - click to specify', select 'Sequences' (top row), and under 'SEQUENCES', click 'Flank-Gene' and select 'Upstream Flank' to fill in an intended length of promoter region. Finally, click 'Results' (top menu).

2 Design a pair of specific primers to amply the intended target of ~500 bp. Order them via the user's preferred oligo synthesis service.

3 Isolate genomic DNA from ~100 mg of plant tissues of interest by using a DNeasy plant pro kit (Qiagen) and following the manufacturer's instructions. Adjust the DNA concentration to ~100 ng/μl by using ddH_2O.

■ **PAUSE POINT** The genomic DNA can be stored at either −20 or −80 °C for ≥1 year.

4 Prepare a PCR reaction to amplify the intended target regions by following thereaction mixture and reagent order below:

Component	Stock concentration	Volume (μl)	Final concentration
Q5 reaction buffer	5×	10	1×
dNTPs	10 mM	1	200 μM
Forward primer from Step 2	10 μM	2.5	0.5 μM
Reverse primer from Step 2	10 μM	2.5	0.5 μM
Template DNA from Step 3	100 ng/μl	1	2 ng/μl
Q5 high-fidelity DNA polymerase	2 U/μl	0.5	0.02 U/μl
Nuclease-free water	-	≤50	-

▲ **CRITICAL STEP** The user should use high-fidelity DNA polymerases, which allow for ultra-low error amplification.

5 Mix the reaction by gently vortexing and then quickly spin all liquid to the bottom of the tubes. Transfer the PCR tube to a thermocycler with the following cycling conditions:

Cycle no.	Denature	Anneal	Extension
1	98 °C, 30 s	-	-
2–33	98 °C, 5 s	Tm °C, 10 s	72 °C, 20 s
34	-	-	72 °C, 5 min

▲ **CRITICAL STEP** The melting temperature (Tm) represents the Tm value of target-specific primer sequences.

6 Load 50 µl of PCR product mixed with 10 µl of 6× loading dye, as well as a DNA ladder in 1% (wt/vol) agarose gel (supplemented with 1:10,000 (vol/vol) ethidium bromide), and run in 1× TAE buffer at 150 V for 25 min.

7 Visualize the gel by using a gel imaging system and cut out the band with the expected size and purify by using the QIAquick PCR purification kit (Qiagen) according to the manufacturer's instructions. **? TROUBLESHOOTING**

8 Dissolve the collected product in water or EB buffer and sequence by using Sanger sequencing. **■ PAUSE POINT** The purified DNA can be stored at −20 °C for 6 months.

9 Compare the sequence obtained via Sanger sequencing and the retrieved sequence from the genome database.
▲ CRITICAL STEP The sequence obtained by Sanger sequencing might be different from the retrieved sequence. Design sgRNAs (see the next steps) on the basis of the result of Sanger sequencing.

Selection of CRISPR-Combo systems and design of sgRNAs ● Timing ~3 h

▲ CRITICAL To ensure successful genome engineering, selecting the appropriate CRISPR-Combo system application based on your purposes is critical before starting to design sgRNAs. The Cas9-Act3.0 can be used for orthogonal DSB-based genome editing[7] and gene activation (Fig. 1a). The editing outcomes of Cas9-Act3.0 are typically unpredictable. By contrast, the CBE-Cas9n-Act3.0 enables precise C-to-T base editing[14] and simultaneous gene activation (Fig. 1b).

10 Select the appropriate CRISPR-Combo system. Generally, we recommend using the Cas9-Act3.0 for targeted gene knockout by editing the coding regions. In addition, it also can be used for deletion of non-coding regions like promoters and introns. CBE-Cas9n-Act3.0 can be used to install C-to-T mutations within the editing windows that may be used for gene knockout through introduction of stop codons.

11 On the basis of the selection of CRISPR-Combo systems, the user needs to design sgRNAs for targeted gene activation manually (option A), DSB-mediated genome editing by using gRNA design tools (option B) or manually for base editing (option C) (Fig. 2a).

(A) **Design sgRNAs for gene activation ● Timing 1 h**

(i) Follow the procedures outlined in Box 1 to design sgRNAs with 15-nt protospacers for targeted gene activation.
▲ CRITICAL STEP Only truncated protospacers of 14–16 nt can be used for CRISPR-Combo-mediated gene activation[23]. We recommend pre-screening sgRNA efficiency by using transient expression systems such as the protoplast system, which has been described in a previously published protocol[19,70]. Then, the most efficient sgRNA can be selected for constructing the multiplexed sgRNA expression vector. Alternatively, the user can design two or three sgRNAs for a target gene activation by following the rules in Box 1.

(B) **Design sgRNAs for DSB-mediated genome editing ● Timing 1 h**

(i) Use the webtool CRISPR-P v2.0 (http://crispr.hzau.edu.cn/CRISPR2/) to design sgRNAs for DSB-mediated genome editing, which is sufficient for most applications. Select the target genome and input the gene locus or genomic DNA sequence of interest.

(ii) Run this program based on the default CRISPR design parameters including PAM, RNA scaffold, and protospacer length.

(iii) On the basis of the scoring system, select sgRNAs showing high on-targeting and minimal off-targeting efficiency.
▲ CRITICAL STEP We recommend choosing sgRNAs targeting early exons for genetic knockout.

(C) **Design sgRNAs for base editing ● Timing 1 h**

(i) Follow the process outlined in Experimental design to manually design sgRNAs with 20-nt protospacers for base editing. Briefly, to manually design sgRNAs, identify available NGG PAMs within the target genomic region. The PAMs should enable the installation of the C-to-T conversion within the optimal editing window (typically the 4th to 15th base pairs upstream of PAM) of CBE-Cas9n-Act.3.0.

(ii) Predict the off-target sites of selected sgRNAs by using Cas-OFFinder[64] (http://www.rgenome.net/cas-offinder/). Select the sgRNAs with minimum off-target potential if multiple NGG PAMs are available.
▲ CRITICAL STEP Ensure that the intended target edits fall within the optimal editing window of the CBE-Cas9n-Act3.0.

Construction of multiplex sgRNA expression vectors

12 Construct multiplex sgRNA expression systems, either M–(At)U3–sgRNA (option A, Fig. 3a) or M–tRNA–sgRNA (option B, Fig. 3b).

▲ **CRITICAL STEP** In the M–U3–sgRNA strategy, a Pol III AtU3 promoter is used to express a single sgRNA with an AtU3 terminator[19,23], and up to six U3–sgRNA expression cassettes can be assembled into an sgRNA array by using the Golden Gate method. By contrast, for the M–tRNA–sgRNA strategy, an endogenous tRNA-processing system is used to produce numerous sgRNAs by precisely cleaving both ends of the tRNA precursor[82]. By using the Golden Gate method, up to six tRNA–sgRNA architectures can be assembled into a single transcript driven by a Pol II promoter, ZmUbi[19,23]. We recommend selecting the M–U3–sgRNA (option A) and M–tRNA–sgRNA (option B) strategies to generate multiplex sgRNA expression vectors in dicot or monocot species, respectively. The user also can use both of them concurrently to figure out which one performs better in their target species or switch the promoter for sgRNA expression on the basis of the plant species preference.

(A) **Construction of multiplex sgRNA expression vector M–U3–sgRNA** ● **Timing 7 d**

(i) Add overhang nucleotides 5′-GGTC-3′ and 5′-AAAC-3′ at the 5′ end of forward and reverse gRNA sequences (Table 2), respectively (Fig. 3a).

▲ **CRITICAL STEP** Because the AtU3 promoter is used in the M–U3–sgRNA system, the 5′ end of gRNAs should start with a DNA base 'A' for efficient gRNA expression. An extra 'A' should be added at the 5′ end of gRNA if its first nucleotide is not 'A' (Table 2).

(ii) Order the forward and reverse gRNA oligos at a concentation of 100 μM by using a desalted purification method.

■ **PAUSE POINT** Resuspended or powdered oligos can be stored at −20 °C for ≥1 year.

(iii) Phosphorylate synthesized gRNA oligos by using T4 PNK at 37 °C for 30 min as follows:

Reagent	Stock concentration	Volume (μl)	Final concentration
gRNA forward oligo	100 μM	1	10 μM
gRNA reverse oligo	100 μM	1	10 μM
T4 PNK reaction buffer	10×	1	1×
T4 PNK	20 U	0.5	1 U
Nuclease-free water	-	≤10	-

(iv) Incubate the mixed reactions at 37 °C for 30 min and then place the reactions in a 1-liter beaker with 300 ml of boiling water to anneal phosphorylated gRNA oligos. Cool the boiling water down to ~30 °C.

! **CAUTION** Wear heat-protection gloves because the beaker with boiling water is hot.

■ **PAUSE POINT** The duplexed oligos can be stored at −20 °C for a few months.

(v) Linearize U3–sgRNA entry plasmids (pYPQ131B/B2.0 to pYPQ13**N**B/B2.0; **N** represents the total number of gRNA sites, up to six) separately by using *Bsm*BI (*Esp*3I) at 37 °C for 2 h and following the instructions shown below.

Reagent	Stock concentration	Volume (μl)	Final concentration
pYPQ131B/B2.0 to 13**N**B/B2.0	100 ng/μl	15	30 ng/μl
Buffer Tango	10×	5	1×
DTT	10 mM	5	1 mM
*Esp*3I (*Bsm*BI)	10 U/μl	2	0.4 U/μl
Nuclease-free water	-	≤50	-

▲ **CRITICAL STEP** The vectors pYPQ131B to pYPQ136B contain the canonical sgRNA scaffold gR1.0 used for genome editing, whereas pYPQ131B2.0 to pYPQ136B2.0 contain an engineered sgRNA scaffold, gR2.0, for gene activation. Thus, the user needs to clone duplexed gRNA oligos into pYPQ131B to pYPQ13**N**B for genome editing and into pYPQ131B2.0 to pYPQ13**N**B2.0 for gene activation.

Table 3 | Construction of multiplex sgRNA expression vector M-U3-sgRNA

Entry plasmids	Number of gRNA sites				
	2	3	4	5	6
pYPQ131B/B2.0	o	o	o	o	o
pYPQ132B/B2.0	o	o	o	o	o
pYPQ133B/B2.0		o	o	o	o
pYPQ134B/B2.0			o	o	o
pYPQ135B/B2.0				o	o
pYPQ136B/B2.0					o
Gateway-compatible entry vector	pYPQ142	pYPQ143	pYPQ144	pYPQ145	pYPQ146

▲ **CRITICAL STEP** To construct the M–U3–sgRNA array, the user must start from pYPQ131B/B2.0 (see Table 3 for details).

(vi) Purify the digested plasmids by using the QIAquick gel extraction kit (Qiagen) according to the manufacturer's instructions.
▲ **CRITICAL STEP** We recommend using gel extraction instead of PCR clean-up for digested product purification to minimize the unintentional collection of undigested or partly digested plasmids.
■ **PAUSE POINT** The purified linearized plasmids can be stored at −20 °C for 6 months.

(vii) Dilute annealed gRNA oligos from Step 12A(iv) at a 1:200 ratio by using sterile ddH_2O and ligate the diluted oligos into linearized sgRNA entry plasmids separately by following the instructions shown below.

Reagent	Stock concentration	Volume (μl)	Final concentration
*Bsm*BI linearized plasmid (Step 12A(vi))	20 ng/ul	1	5 ng/ul
Diluted annealed oligos (Step 12A(iv))	0.05 μM	1	0.0125 μM
Instant sticky-end ligase master mix	2×	2	1×

(viii) Incubate the reactions at 22 °C for 15 min and transform 4 μl into 50 μl of *E. coli* DH5α competent cells by using the heat-shock method. Briefly, incubate the mixture on ice for 10 min, heat-shock at 42 °C for 50 s, place back to ice immediately for 2 min, incubate the mixture with 500 μl of LB broth in a 37 °C shaking incubator at 220 rpm for 1 h, collect the cells by centrifugation at 2,500*g* at 25 °C for 1 min, discard most of the supernatant and resuspend the cells in residual LB solution (~100 μl).

(ix) Spread all resuspended cells onto one LB solid plate supplied with tetracycline (10 μg/ml) and incubate the plates at 37 °C overnight.
▲ **CRITICAL STEP** The vectors pYPQ131B/B2.0 to pYPQ136B/B2.0 use tetracycline resistance as the selectable marker. Therefore, prepare the LB solid plates supplemented with tetracycline (10 μg/ml).

(x) Pick two colonies by using sterile 10-μl tips for each vector and incubate each overnight in 8 ml of LB supplemented with tetracycline (10 μg/ml) by using a 15-ml culture tube at 37 °C. Isolate plasmid DNA by using a Hi-speed mini plasmid kit (IBI) or other equivalent kit and following the manufacturer's instructions.
■ **PAUSE POINT** The plasmid DNA can be stored at −20 °C for 1 year.
? TROUBLESHOOTING

(xi) Following the instructions of the sequencing service (e.g., GENEWIZ), confirm gRNA insertion by using Sanger sequencing with primer pTC14−F2 (Table 2).

(xii) To construct a multiplexed sgRNA expression vector, simultaneously assemble the pYPQ131B/B2.0 to pYPQ13**N**B/B2.0 plasmids into a single Gateway-compatible entry vector pYPQ14**N** by using Golden Gate assembly, according to the following instructions.

Reagent	Stock concentration	Volume (μl)	Final concentration
pYPQ131B/B2.0 to pYPQ13**N**B/B2.0	100 ng/μl	1	5 ng/μl
pYPQ14**N**	100 ng/μl	1	5 ng/μl
T4 DNA ligase buffer	10×	2	1×
*Bsa*I-HF v2	20 U/μl	1	1 U/μl
T4 DNA ligase	400 U/μl	1	20 U/μl
Nuclease-free water	-	≤20	-

▲**CRITICAL STEP** The letter '**N**' in pYPQ13**N**B/B2.0 and pYPQ14**N** indicates the total number of gRNA sites, up to six. For example, the user should select pYPQ131B/B2.0, pYPQ132B/B2.0, pYPQ133B/B2.0, pYPQ134B/B2.0 and pYPQ144 if four gRNAs are designed for co-expression.
▲**CRITICAL STEP** To set up a control for the CRISPR-Combo vector, the sgRNA entry plasmids without gRNA insertion should be performed for Golden Gate assembly concurrently. Alternatively, other control vectors for editing or activation only may be constructed in this step.

(xiii) Perform the Golden Gate reactions under the following conditions by using a thermocycler.

Step	Temperature (°C)	Time (min)	Cycle
1	37	5	10 (Step 1 + Step 2)
2	16	10	-
3	50	5	1
4	80	5	1

(xiv) Transform the 20 μl of reaction mixture into *E. coli* DH5α competent cells by using the heat-shock method (as described in Step 12A(viii)).

(xv) Pour 70 μl of 20 mg/ml X-gal along with 70 μl of 0.1 M IPTG onto the LB solid medium supplemented with spectinomycin (50 μg/ml), spread evenly by using sterilized plating beads and dry in a laminar flow hood for 10 min; then, spread the resuspended cells from Step 12A(xiv) onto the LB plate by using plating beads and incubate the plates at 37 °C overnight.
▲**CRITICAL STEP** The IPTG is used by coupling with X-gal for blue-white screening, which is an effective molecular biology method to screen for the recombinant vector of pYPQ14**N**. A white colony indicates the desired clone with successful recombination of pYPQ14**N** and pYPQ131B/B2.0 to pYPQ13**N**B/B2.0. By contrast, a blue colony represents a negative clone.

(xvi) Repeat Step 12A(x) to pick and grow colonies with spectinomycin (50 μg/ml) and isolate plasmid DNA.
? TROUBLESHOOTING

(xvii) Confirm the successful Golden Gate reaction by digesting the plasmids by using both restriction enzymes *Bam*HI-HF and *Xho*I. Prepare the reaction as follows:

Reagent	Stock concentration	Volume (μl)	Final concentration
Plasmids	100 ng/μl	10	20 ng/μl
*Bam*HI-HF	20 U/μl	1	0.4 U/μl
*Xho*I	20 U/μl	1	0.4 U/μl
CutSmart Buffer	10×	5	1×
Nuclease-free water	-	≤50	-

(xviii) Incubate the reaction at 37 °C for 1 h and follow the same process as Step 6 to run the gel. Two bands should be detected. One is 1,433 bp, and the other one is 1,400 bp plus the size of the inserted sgRNA cassettes.

Table 4 | Construction of multiplex sgRNA expression vector M-tRNA-sgRNA

Entry plasmids	Number of sgRNA sites				
	2	3	4	5	6
pYPQ131-tRNA/-tRNA2.0	o	o	o	o	o
pYPQ132-tRNA/-tRNA2.0	o	o	o	o	o
pYPQ133-tRNA/-tRNA2.0		o	o	o	o
pYPQ134-tRNA/-tRNA2.0			o	o	o
pYPQ135-tRNA/-tRNA2.0				o	o
pYPQ136-tRNA/-tRNA2.0					o
Gateway-compatible entry vector	pYPQ142-ZmUbi-tRNA	pYPQ143-ZmUbi-tRNA	pYPQ144-ZmUbi-tRNA	pYPQ145-ZmUbi-tRNA	pYPQ146-ZmUbi-tRNA

(xix) Following the instructions of the selected sequencing service (e.g., GENEWIZ), confirm sgRNA cassette insertion by using Sanger sequencing with primers M13−F, M13−R and forward or reverse gRNA oligos (Table 2).

PAUSE POINT The plasmid DNA can be stored at −20 °C for 1 year.

(B) **Construction of multiplex sgRNA expression vector M−tRNA–sgRNA** ● **Timing 7 d**

CRITICAL Unlike the M–U3–sgRNA system, which requires the 5′ end of gRNAs to be the base 'A', there is no such requirement for gRNAs used in the M−tRNA–sgRNA system, because a Pol II promoter ZmUbi is used for sgRNA expression (Fig. 3b).

(i) Add overhang nucleotides 5′-TGCA-3′ and 5′-AAAC-3′ at the 5′ end of forward and reverse gRNA oligos (Table 2), respectively.

(ii) Repeat Step 12A(ii–iv) to synthesize, phosphorylate and anneal gRNA oligos, Step 12A(v and vi) to linearize tRNA–sgRNA entry plasmids, pYPQ131-tRNA/-tRNA2.0 to pYPQ13**N**-tRNA/-tRNA2.0 (**N** represents the total number of gRNA sites, up to six), Step 12A(vii–ix) to clone gRNA sequences into the linearized sgRNA entry plasmids and Step 12A(x and xi) to miniprep and sequence these plasmids. Repeat Step 12A(xii) to construct a multiplexed sgRNA expression vector by simultaneously assembling the pYPQ131-tRNA/-tRNA2.0 to pYPQ13**N**-tRNA/-tRNA2.0 into a single Gateway-compatible entry vector, pYPQ14**N**-ZmUbi-tRNA, by using Golden Gate assembly (see Table 4), as described in Step 12A(xiii–xvi). Repeat Step 12A(xvii and xviii) to confirm the successful Golden Gate reaction by digesting the plasmids by using both restriction enzymes *Bam*HI-HF and *Eco*RI-HF. Two bands should be detected. One has a length of 4,776 bp, and the other one is the size of the inserted tRNA-sgRNA cassettes. Following Step 12A(xix), confirm sgRNA cassette insertion with primers Ubi−intron−F1, M13−R1 or both on the basis of the length of insertion (Table 2).

CRITICAL STEP The vectors pYPQ131-tRNA/-tRNA2.0 to pYPQ136-tRNA/-tRNA2.0 and pYPQ14**N**-ZmUbi-tRNA use tetracycline (10 μg/ml) resistance and spectinomycin (50 μg/ml) resistance as the selectable markers, respectively.

PAUSE POINT The plasmid DNA can be stored at −20 °C for 1 year.

Constructing CRISPR-Combo T-DNA vectors by Gateway cloning ● **Timing 3 d**

13 After confirmation of the multiplex sgRNA expression vector by sequencing, mix the correct sgRNA plasmid with Cas9-Act3.0 or CBE-Cas9n-Act3.0 and the destination vector pYPQ202 (dicots) or pYPQ203 (monocots) to perform the LR Gateway reaction, as follows:

Reagent	Stock concentration	Volume (μl)	Final concentration
Cas9-Act3.0 or CBE-Cas9n-Act3.0	100 ng/μl	1.5	30 ng/μl
Multiplex sgRNA expression vector (Step 12)	100 ng/μl	1.5	30 ng/μl
Destination vector pYPQ202 or pYPQ203	200 ng/μl	1	40 ng/μl
LR clonase II enzyme mix	5×	1	1×

CRITICAL STEP In this protocol, the destination vectors pYPQ202 and pYPQ203 are chosen for dicot and monocot species, respectively. The vector pYPQ202 contains the promoter AtUBQ10 to

drive the expression of Cas9 or CBE-Cas9n protein, whereas pYPQ203 uses the promoter ZmUbi for Cas9 or CBE-Cas9n expression. Both destination vectors can confer hygromycin resistance to transgenic plants. The user may replace the promoter in either destination vector with a promoter of interest or use other equivalent destination vectors with the attR1 and attR2 recombination sites.
▲ **CRITICAL STEP** The control T-DNA vector should be generated through the LR Gateway reaction concurrently.

14 Incubate the reactions at 25 °C for ≥3 h or overnight (recommended). Then, transform the reaction mixture into *E. coli* DH5α competent cells by using the same procedures as in Step 12A(viii).

15 Spread the cells onto LB solid plates supplied with kanamycin (50 μg/ml) and incubate the plates at 37 °C overnight.
▲ **CRITICAL STEP** The vectors pYPQ202 and pYPQ203 use kanamycin resistance as the selectable marker. Therefore, prepare the LB solid plates supplemented with kanamycin (50 μg/ml).

16 Repeat Step 12A(x) to pick and grow colonies with kanamycin (10 μg/ml) and isolate plasmid DNA.
■ **PAUSE POINT** The plasmid DNA can be stored at −20 °C for 1 year.
? TROUBLESHOOTING

17 Repeat Step 12A(xvii) to confirm the successful LR Gateway reaction by using both restriction enzymes *Eco*RI-HF and *Hin*dIII-HF.

18 Incubate the reaction at 37 °C for 1 h and follow the same process as Step 6 to run the gel. Analyze the digested bands by using preferred analysis software (e.g., SnapGene viewer).

19 Prepare glycerol stock for the correct bacterial strains on the basis of the electrophoresis results from Step 18. Mix equal volumes (500 μl) of sterilized 50% (vol/vol) glycerol and bacterial culture from the Step 16 well.
■ **PAUSE POINT** The glycerol stock can be stored at −80 °C for ≥1 year.

Part 2: speed breeding of transgene-free, genome-edited *Arabidopsis* plants by using CRISPR-Combo

Transformation of *Agrobacterium* with CRISPR-Combo T-DNA vector ● **Timing 7 d**

20 Using the freeze-thaw method[83], transform the CRISPR-Combo T-DNA vector from Step 18 into *A. tumefaciens* GV3101 or EHA105 for *Arabidopsis* and rice transformation, respectively. See Fig. 2b.
▲ **CRITICAL STEP** The control T-DNA vector system should be transformed concurrently.

21 To avoid selecting the *Agrobacterium* colony with undesirable T-DNA recombination, pick two colonies by using sterile 10-μl tips for each vector and incubate each for 48 h in 8 ml of LB supplemented with both kanamycin (10 μg/ml) and rifampicin (50 μg/ml) by using 15-ml culture tubes. Isolate plasmid DNA by using a Hi-speed mini plasmid kit (IBI) or other equivalent kit and following the manufacturer's instructions.
▲ **CRITICAL STEP** Ensuring that DNA plasmids have not been recombined in *Agrobacterium* is critical because the T-DNA vector contains many repetitive homologous sequences of tRNA, U3 or sgRNA scaffold. We recommend isolating plasmids from *Agrobacterium* and re-transforming them into *E. coli* DH5α competent cells to screen for the correct *Agrobacterium* colony without T-DNA rearrangement by following Steps 22–25.
? TROUBLESHOOTING

22 Transform the 50 ng of extracted plasmids from Step 21 into *E. coli* DH5α competent cells by following the same procedures as in Step 12A(viii).

23 Spread the cells onto LB solid plates supplied with kanamycin (50 μg/ml) and incubate the plates at 37 °C overnight.

24 Pick three colonies by using sterile 10-μl tips for each vector and incubate each overnight in 8 ml of LB supplemented with kanamycin (10 μg/ml) by using 15-ml culture tubes. Isolate plasmid DNA by using a Hi-speed mini plasmid kit (IBI) or other equivalent kit and following the manufacturer's instructions.

25 Digest the extracted plasmids from Step 24 by using both restriction enzymes *Eco*RI-HF and *Hin*dIII-HF and following the descriptions in Steps 17 and 18.

26 Select the correct *Agrobacterium* colony without T-DNA recombination and prepare a glycerol stock as described in Step 19 for subsequent *Arabidopsis* transformation (Step 27) or rice transformation (Step 57).
■ **PAUSE POINT** The glycerol stock can be stored at −80 °C for ≥1 year.

Floral dip transformation of *Arabidopsis* plants ● Timing 4 d

27 Deliver CRISPR-Combo T-DNA from Step 26 and related control T-DNA into *Arabidopsis* via *Agrobacterium*-mediated floral dip transformation[72].

Identification of T_1 Combo *Arabidopsis* plants via flowering phenotype ● Timing 3 weeks

28 Sterilize T_1 seeds harvested from Step 27 by using 50% (vol/vol) bleach mixed with 0.05% (vol/vol) Tween 20 in a 15-ml centrifuge tube, shaking the tube for 10 min. Then, centrifuge at 1,000*g* for 30 s and remove the supernatant. Rinse the seeds five times by using sterile water.
▲ **CRITICAL STEP** Sterilization of the T_1 seeds from the control T-DNA system should be performed concurrently.

29 Resuspend the sterilized seeds from Step 28 in 8 ml of sterile 0.05% (wt/vol) agarose and keep them at 4 °C for 3 d.
▲ **CRITICAL STEP** Keeping seeds at 4 °C allows for vernalization, ensuring seed germination and physiological state uniformity.

30 Pour ~4 ml of the seed-agarose suspension onto a 100 × 15 mm 1/2 MS plate supplemented with hygromycin (15 μg/ml) and Timentin (100 μg/ml). Spread the seed suspension equally on the plate. Move the plates to a growth chamber under a long-day condition (16 h of light/8 h of dark) at 22 °C.
▲ **CRITICAL STEP** The Timentin is necessary for restraining *Agrobacterium* contamination from seeds.

31 After ~7 d, transgenic Combo and control lines should be distinguished as seedlings that show healthy green cotyledons and true leaves and developed roots. By contrast, the cotyledons of non-transformed seedlings remain green; however, their size is significantly smaller than that of true transformants. In addition, the non-transformed seedlings cannot develop true leaves and long roots. Transfer those potential transgenic Combo and control lines to fresh 1/2 MS plates containing Timentin (100 μg/ml) for recovery.
? TROUBLESHOOTING

32 After another 7 d, flower buds emerge in transgenic Combo populations (~30–50%) after those seedlings develop two true leaves (Fig. 2b). Those plants, which we call 'extra-early-flowering plants', produce flower buds with only four leaves. In control lines, no such flowering phenotype is observed.
▲ **CRITICAL STEP** In the Combo systems, there is a highly positive correlation between the targeted genome editing and gene activation activity[23]. The extra-early-flowering phenotype results from Combo-mediated high activation of the florigen gene *AtFT*. Therefore, the editing activity of extra-early-flowering plants is higher than other wild type-like plants. The flowering phenotype can be used as a selection marker to enrich and visually screen plants with high activity of gene activation and genome editing.
▲ **CRITICAL STEP** Besides the extra-early-flowering plants, there will be early-flowering and standard flowering plants in the Combo population. In general, early-flowering and standard flowering plants show 6–18 and 20–25 leaves, respectively, when flower buds become visible. Both early-flowering and standard flowering plants contain lower gene activation and genome-editing activity than those of extra-early-flowering plants[23].
? TROUBLESHOOTING

33 Transfer these extra-early-flowering Combo and related control plantlets to soil and grow them in a growth chamber under a long-day condition (16 h of light/8 h of dark) at 22 °C.

Determining the genome-editing efficiency of extra-early-flowering Combo plants ● Timing 4 weeks
▲ **CRITICAL** To identify the targeted mutations of selected Combo lines by using NGS, a high-throughput Hi-TOM method[74] is used in this protocol (see Experimental design).

34 Using the CTAB method[84], extract genomic DNA from 50 mg of leaf tissue or one leaf for each plant. Dilute the extracted DNA to a final concentration of 100 ng/μl by using ddH_2O.
■ **PAUSE POINT** DNA samples can be stored at −20 °C for ≥1 year.

35 Design specific PCR primers to amplify the target region and then fuse common bridging sequences 5′-ggagtgagtacggtgtgc-3′ and 5′-gagttggatgctggatgg-3′ to the 5′ end of forward and reverse primers (Table 1), respectively, resulting in Hi-TOM primers for the first round of PCR (PCR-1). The common bridging sequence can be recognized by the Hi-TOM common primers used in the second PCR (PCR-2) (Table 1).
▲ **CRITICAL STEP** The amplicon size can be designed as ~300 bp, and its length should follow the requirement of selected sequencing services (e.g., GENEWIZ, 150–500 bp using Illumina 2 × 250 bp platform). Ensure that the target site resides around the center of the amplicon.

36 Using a highly efficient and high-fidelity DNA polymerase (e.g., Phire plant direct PCR kit), perform a PCR-1 reaction to amplify the target region as follows:

Component	Stock concentration	Volume (μl)	Final concentration
2× Phire plant PCR buffer	2×	10	1×
Hi-TOM-F	5 μM	2	0.5 μM
Hi-TOM-R	5 μM	2	0.5 μM
Template DNA	100 ng/μl	0.5	2.5 ng/μl
Phire hot start II DNA polymerase	-	0.2	-
ddH_2O	-	≤20	-

37 Perform PCR-1 by using the following cycling conditions:

Cycle no.	Denature	Anneal	Extension
1	98 °C, 5 min	-	-
2–15	98 °C, 5 s	Tm °C, 5 s	72 °C, 20 s
16	-	-	72 °C, 1 min

▲ **CRITICAL STEP** The Tm represents the Tm value of target-specific primer sequences without the common bridging sequences, which can be predicted by using a primer design tool or a web-based Tm Calculator.

▲ **CRITICAL STEP** We recommend evaluating the specificity of PCR-1 primers by using the shown conditions but with a 35-cycle amplification. Optimize the primers with different Tm values or test different pairs of primers to ensure that only the desired band is produced.

? TROUBLESHOOTING

38 Premix the 5 μM stocks of Hi-TOM common primers for the PCR-2 in a standard 96-well PCR plate. Load the forward Hi-TOM primers Hi-TOM-F1 to Hi-TOM-F12 in columns 1–12 and the reverse Hi-TOM primers Hi-TOM-R-A to Hi-TOM-R-H in rows A–H.

▲ **CRITICAL STEP** Besides the bridging and barcode sequences, an NGS platform-specific adapter sequence is also added to the 5′ end of each primer (Table 1). In this protocol, the adapter sequences are designed on the basis of the instructions from GENEWIZ.

39 Perform the PCR-2 reaction via re-amplifying the PCR-1 products by using Hi-TOM common primers as follows:

Component	Stock concentration	Volume (μl)	Final concentration
2× Phire plant PCR buffer	2×	20	1×
Hi-TOM primers mixture	5 μM (per primer)	4	0.5 μM (per primer)
PCR-1 template	-	0.5	-
Phire hot start II DNA polymerase	-	0.5	-
ddH_2O	-	≤40	-

40 Perform PCR-2 by using the following cycling conditions:

Cycle no.	Denature	Anneal	Extension
1	98 °C, 5 min	-	-
2–35	98 °C, 5 s	61 °C, 5 s	72 °C, 20 s
36	-	-	72 °C, 1 min

The annealing temperature represents the Tm value of common bridging sequences.

41 Run 5 μl of PCR-2 products on a 1% (wt/vol) agarose gel at 150 V for 25 min with a DNA ladder. PCR products should be visible at the expected band size of the amplicon plus 120 bp (bridging, barcode and adapter sequences).

▲ CRITICAL STEP Multiple amplification bands might be produced for one sample even if the PCR-1 primers have been optimized. We found that the subsequent sequencing coverage of the desired amplicon works well if the density of designed amplicons is generally over 50% of the total bands. If the percentage of desired band is lower than 50%, we recommend optimizing the Tm value for PCR-2 primers or using new primers for PCR-1.

? TROUBLESHOOTING

42 Pool PCR-2 products (up to 96) together into one PCR product mixture. Purify the mixture by using QIAquick PCR & gel cleanup kit (Qiagen) according to the manufacturer's instructions. Elute the purified PCR-2 mixture in 100 µl of ddH_2O.

▲ CRITICAL STEP The number of sequencing reads for each sample is negatively correlated with the number of pooled samples. In general, more than 1,000 sequencing reads can be produced for each sample if 96 PCR-2 samples are pooled into one NGS sample. The number of pooled samples should be adjusted according to the desired sequencing coverage and quality of PCR-2 amplicons.

■ PAUSE POINT The purified DNA can be stored at −20 °C for 1 year.

43 Prepare the NGS samples by following the instructions for amplicon sequencing (e.g., GENEWIZ and Amplicon-EZ) and send them for sequencing. In general, it takes a few weeks to get the sequencing results.

44 When completed, two zipped fastq files, R1.fastq.gz and R2.fastq.gz, are produced for each NGS sample. Following the instructions for CRISPRMatch software[77], merge the paired-end reads into single-end reads. Then, split these single-end reads into individual PCR-2 amplicons by using the designed barcodes.

45 Following the instructions of webtool CRISPResso2 (ref. [75]) (http://crispresso.pinellolab.org/submission) or CRISPR RGEN (http://www.rgenome.net/), analyze the editing outcomes by using the individual single-end reads for each PCR-2 amplicon.

▲ CRITICAL STEP The read number is provided in the output files for each amplicon. Those amplicons with a low sequencing depth (too few reads, e.g., <200 reads) may lead to biases in downstream analyses.

? TROUBLESHOOTING

46 On the basis of the editing outcomes from Step 45, identify the Combo plants with desired mutations and obtain their progenies by selfing.

Screening transgene-free genome-edited plants via flowering phenotype ● Timing 6 weeks

47 Harvest the seeds from the labeled Combo plants from Step 46 individually.

▲ CRITICAL STEP The extra-early-flowering Combo plants have a significantly shorter life cycle[23], which needs only ~40 d to produce seeds. Correspondingly, their seed yield reduces to ~20% of that of wild-type plants, which is still enough for the following steps.

48 Suspend the seeds of Combo and control lines separately in sterile 0.05% (wt/vol) agarose and keep them at 4 °C for 3 d to break dormancy. Then, plant the seeds into soil by using a 50-cell seed tray.

49 Move the seed tray to a growth chamber and grow these plants under a long-day condition (16 h of light/8 h of dark) at 22 °C. Allow them to grow for 6 weeks; three categories of plants, extra-early-flowering, early-flowering and standard flowering plants, will emerge.

50 To save space, discard the extra-early-flowering and early-flowering plants in a timely manner on the basis of the number of leaves and flowering time.

▲ CRITICAL STEP The extra-early-flowering, early-flowering and standard flowering plants generally show 4, ~6–18 and ~20–25 leaves when flower buds become visible. The control plants can be used as an indicator to distinguish the early-flowering and standard flowering plants. Generally, 25% of the total population of each line are standard flowering plants[23].

51 Extract the genomic DNA from the remaining standard flowering plants by following Step 34.

52 Repeat Steps 4–6 by using two pairs of primers (Table 2) targeting the upstream and downstream region of the CRISPR-Combo T-DNA vector, respectively, to identify the transgene-free, genome-edited plants.

▲ CRITICAL STEP The first pair of custom primers can amplify the Cas9-SunTag region within the T-DNA vector at a length of 2,329 bp. For the second pair of custom primers, we recommend using one forward sgRNA coupled with a reverse sgRNA oligo to amplify the sgRNA expression cassette. Use sterile ddH_2O as a negative control.

▲ CRITICAL STEP On average, >90% of standard flowering plants should be transgene free[23].

? TROUBLESHOOTING

53 Analyze the genotypes of the transgene-free plants identified from Step 52 by following Steps 35–45. Collect the seeds from transgene-free plants with the desired mutations for further applications.

Part 3: enhancing rice regeneration and heritable mutations in an HF manner by using CRISPR-Combo

***Agrobacteria*-mediated transformation of rice calli with the CRISPR-Combo construct for HF tissue culture and genome editing ● Timing 4 months**

▲ **CRITICAL** The protocol for *Agrobacteria*-mediated transformation of rice calli mainly refers to a previous report[79] with related modifications to develop genome-edited rice plants in an HF manner. Detailed procedures for *Agrobacteria*-mediated transformation of rice calli can be found in the original publication[79].

54 Dehusk mature rice seeds manually or by using a rice husker. Pre-rinse rice seeds in 30 ml of 70% (vol/vol) ethanol for 1 min and then rinse seeds by using sterile ddH_2O once, followed by surface-sterilization of seeds in 40 ml of 50% (vol/vol) bleach for 30 min with vigorous shaking using a shaker. Finally, rinse seeds five times by using sterile ddH_2O. See Fig. 2c.

▲ **CRITICAL STEP** Do not damage the embryo of seeds during de-husking. For each vector, we recommend using 50 seeds for calli induction. All operations in this step and Steps 55–62 are performed in a laminar flow hood.

55 Dry the seeds on sterile filter papers and then place 15 sterilized seeds onto rice callus induction medium per plate. Seal plates with micropore surgical tape and incubate the seeds in a culture chamber at 29 °C under a 16 h light/8 h dark photoperiod for 2 weeks.

▲ **CRITICAL STEP** Check the seeds often and transfer uncontaminated seeds onto fresh callus induction medium if any contamination happens.

? TROUBLESHOOTING

56 After 2 weeks, developing calli showing a yellowish color are observed. Four days before the *Agrobacterium* infection, transfer these induced calli to fresh callus induction medium.

57 Streak the *Agrobacterium* colony from Step 26 on LB solid plates supplied with kanamycin (10 μg/ml) and rifampicin (50 μg/ml) and incubate the plates at 28 °C for 3 d.

58 Scrape the *Agrobacterium* cells by using a 1-ml sterilized tip and suspend in 10 ml of rice infection medium supplemented with 100 μM acetosyringone and 1.5 mg/l 2,4-D. Adjust the OD_{600} to 0.4 by using the infection medium.

▲ **CRITICAL STEP** The acetosyringone and 2,4-D are added into the infection medium on the day of calli infection.

59 Collect subcultured calli from Step 56 by using 50-ml sterile centrifuge tubes and pre-wash these calli by using a bacteria-free infection medium supplemented with 100 μM acetosyringone once; then, transfer calli into the *Agrobacterium* cell suspension prepared in Step 58, shaking for 5 min by using a shaker with a low-speed setting.

60 After infection, briefly dry calli on sterilized filter papers and transfer 20 calli per plate onto the surface of co-cultivation medium. Wrap co-cultivation plates by using aluminum foil and incubate at 28 °C for 3 d.

▲ **CRITICAL STEP** No hormone is provided in co-cultivation medium. Excess *Agrobacterium* growth reduces the transformation efficiency.

61 After 3-d co-cultivation, collect calli in 50-ml sterile centrifuge tubes and wash them five times by using sterile ddH_2O supplemented with Timentin (200 mg/l).

▲ **CRITICAL STEP** Rinse the calli with sterile water a few more times if the rinse water is turbid.

62 Dry washed calli on sterile filter paper and place ~35 individual callus pieces onto rice selection medium containing hygromycin (50 mg/l) and 1,000× Timentin (100 mg/l).

▲ **CRITICAL STEP** Ensure that the excess water from calli is removed before transferring to the selection medium. No hormone is supplied in the selection medium.

63 Incubate plates in the light at 32 °C. Sub-culture calli by using a fresh selection medium every 2 weeks.

▲ **CRITICAL STEP** Check the calli often, because the *Agrobacterium* cells may overgrow. Re-wash the *Agrobacterium*-contaminated calli and transfer uncontaminated calli onto fresh selection medium.

? TROUBLESHOOTING

64 For the Combo vector with activation of the morphogenic gene *OsBBM1*, growing calli begin to appear after 6–8 weeks of selection. By contrast, no growing callus is observed in the control (see Controls and Step 20).

▲ **CRITICAL STEP** Each growing callus piece is identified as an independent transgenic event; do not mix these independent transgenic calli. In general, 40% of infected calli are able to produce growing calli.

? TROUBLESHOOTING

65 Transfer independent transgenic events separately to fresh selection medium for another 2 weeks to produce enough tissue for regeneration.

66 After 2 weeks, transfer growing callus tissues onto rice regeneration medium I and incubate in the light at 29 °C for 4 weeks. Sub-culture calli by using a fresh selection medium every 2 weeks.
▲ CRITICAL STEP No hormone is supplied in the regeneration medium I.

67 Transgenic shoots become visible from callus tissues during regeneration. Transfer the ~3-cm-long shoots of rice to regeneration medium II for rooting. Incubate in the light at 29 °C for 2 weeks.
▲ CRITICAL STEP No hormone is supplied in the regeneration medium II. On average, 60–80% of growing calli will enable generation of transgenic shoots, and three to seven transformants can be produced from an independent transgenic event.
? TROUBLESHOOTING

68 Transplant rooted plantlets to soil pots and grow them in a growth chamber at 29 °C.

On- and off-target analysis of genome editing in T_0 Combo rice plants ● Timing 4 weeks

69 Repeat Steps 34–45 to extract genomic DNA from Combo rice plants, design specific PCR-1 primers (Table 1) to amplify the target region and putative off-target loci (see Experimental design) and analyze the editing outcomes and putative off-target events of Combo rice plants.
■ PAUSE POINT DNA samples can be stored at −20 °C for ≥1 year.

? TROUBLESHOOTING

Troubleshooting

Troubleshooting advice can be found in Table 5.

Table 5 | Troubleshooting

Step	Problem	Possible reason	Solution
7	No desired PCR product	Low-quality template DNA	Prepare new genomic DNA by following the manufacturer's instructions
		Low specificity of primers	Re-design a new pair of primers
		High complexity of the proximal promoter sequence	Try new high-fidelity DNA polymerases or a new PCR method like nested PCR based on two pairs of primers
12A(x)	No colonies on the selective plate	Low quality and concentration of the digested backbone vector	Re-digest 2 μg of the backbone vector for 3 h and elute the purified digested plasmids in 30 μl of ddH_2O
		Wrong overhang sequence	Synthesize sgRNAs with correct overhang sequences
	Too many negative colonies	Incomplete digestion of the backbone vector	Re-digest the backbone vector
12A(xvi)	No colonies or too many blue colonies on the selective plate	Low efficiency of restriction enzyme *Bsa*I	Use highly efficient restriction enzyme *Bsa*I-HFv2 (NEB)
		Wrong selection medium	Use the LB medium supplemented with spectinomycin
		Low-quality plasmids	Check for degradation of plasmids and re-miniprep
16	No colonies on the selective plate	Low efficiency of LR clonase enzyme	Ensure that the correct version of clonase enzyme was used. Use new LR clonase II enzyme and store the enzyme kits at −80 °C
		Too short an incubation time	Incubate the reactions at 25 °C overnight
21	Very low yield of plasmid DNA	Low plasmid copy numbers in *Agrobacterium* cells	Use a much larger volume of culture and incubate *Agrobacterium* cells for a longer time
31	No transformants	Wrong selection medium	Use correct antibiotics with an appropriate concentration
		Low efficiency of *A. thaliana* transformation	Follow the published protocol[72]
32	No extra-early-flowering plants	Incorrect design of the Combo vector	Use the published sgRNAs for *AtFT*[23] activation and strictly abide by the protocol to construct the Combo vector
37	No products for PCR-1	Low quality, too high or too low concentration of template DNA	Adjust the DNA concentration to ~100 ng/μl
		Low amplification efficiency or specificity of primers	Re-design highly specific primers by blasting them against the genome of target species

Table continued

Table 5 (continued)

Step	Problem	Possible reason	Solution
41	Multiple amplification bands	Nonspecific primers or too high a concentration of Hi-TOM primers for PCR-2	Increase the annealing temperature, reduce the input of template DNA of PCR-1 or primers for PCR-2. Cut and gel-extract the band at the correct size for the amplicon
45	Too few reads or poorly aligned reads	Low quality of DNA samples and specificity of primers	Reamplify the target region by using a new pair of primers and strictly abide by the manufacturer's instructions to purify PCR products. Reduce the number of samples pooled together
52	No transgene-free plants	Too small a population used for screening or DNA contamination during the PCR process	Screen ≥30 standard flowering plants. Autoclave ddH_2O, tips and pipettes before performing PCR
55	Seed contamination	Insufficient sterilization of seeds	Increase the concentration of bleach, extend the time of sterilization or use a fresh batch of bleach
63	Re-growth of *Agrobacterium* on the surface of calli	Insufficient washing of calli	Embed calli into the selection medium for the first week
64	No new growing calli	Incorrect design of the Combo vector	Use the published sgRNAs for *OsBBM1* (ref. [23]) activation and strictly abide by the protocol to construct the Combo vector
		Unhealthy or low-vigor calli used for transformation	Re-perform the *Agrobacterium*-mediated transformation by using healthy calli
67	No regeneration of the shoot	Wrong regeneration medium or inappropriate culture conditions	Prepare new medium and culture, growing calli under appropriate culture conditions
69	No or low editing activity	Low activity of designed sgRNAs	Pre-screen sgRNA activity in rice protoplasts and use those with high activity

Timing

Steps 1–9, sequencing of target sites: 2 d
Step 10, selection of suitable CRISPR-Combo systems: 10 min
Step 11A, designing sgRNAs for gene activation: 1 h
Step 11B, designing sgRNAs for gene knockout: 1 h
Step 11C, designing sgRNAs for base editing: 1 h
Step 12A, constructing multiplex sgRNA expression systems M–(At)U3–sgRNA: 7 d
Step 12B, constructing multiplex sgRNA expression systems M–tRNA–sgRNA: 7 d
Steps 13–19, constructing CRISPR-Combo T-DNA vectors by Gateway cloning: 3 d
Steps 20–26, transformation of *Agrobacterium* with the CRISPR-Combo T-DNA vector: 7 d
Step 27, floral dip transformation of *Arabidopsis* plants: 4 d
Steps 28–33, identification of T_1 Combo *Arabidopsis* plants via flowering phenotype: 3 weeks
Steps 34–46, determining the genome-editing efficiency of Combo plants: 4 weeks
Steps 47–53, screening transgene-free, genome-edited plants via flowering phenotype and NGS: 6 weeks
Steps 54–61, delivery of CRISPR-Combo T-DNA into rice calli via *Agrobacterium*-mediated transformation: 3 weeks
Steps 62–68, selection and regeneration of Combo rice plants in an HF manner: 14 weeks
Step 69, on- and off-target analysis of genome editing in T_0 Combo rice plants: 4 weeks

Anticipated results

Part 2: speed breeding of transgene-free, genome-edited *Arabidopsis* plants

A representative example of speed breeding of transgene-free, genome-edited plants is given in Fig. 4. Using the CBE-Cas9n-Act3.0 system, we demonstrated that this system enabled simultaneous high-efficiency base editing of two herbicide target genes (*AtALS* and *AtACC2*) with concurrent activation of *AtFT* in *Arabidopsis* plants[23] (Fig. 4a,b). By activation of *AtFT*, ~30–50% of T_1 Combo plants showed an extra-early-flowering phenotype, which enabled shortening the life cycle of *Arabidopsis* plants from 70 to 40 d (Fig. 4a). In addition, the base-editing efficiency of extra-early-flowering plants was significantly higher than that of early-flowering and standard flowering plants (Fig. 4c). In the progeny of the extra-early-flowering T_1 lines, ~25% of standard flowering plants were identified and >90% of them were transgene free (Fig. 4d). Overall, we found that Combo-induced early-flowering phenotype could be reliably used as a phenotypic marker to visually identify T_1 *Arabidopsis* plants with high editing activity and T_2 *Arabidopsis* plants without the transgene.

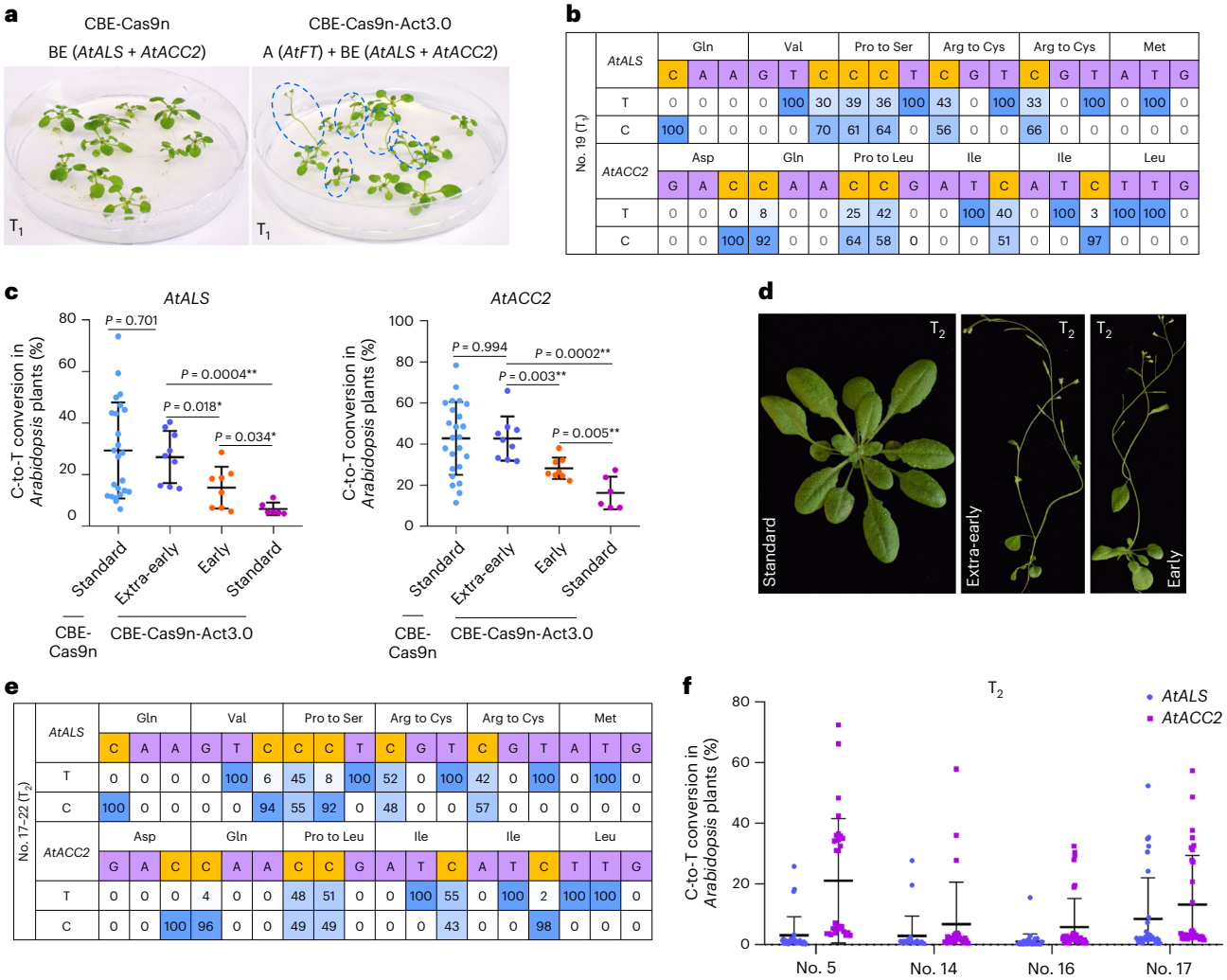

Fig. 4 | Anticipated results of CBE-Cas9n-Act3.0-mediated speed breeding of transgene-free, genome-edited *Arabidopsis* plants via activation of *AtFT*. **a**, Representative images of CBE-Cas9n-Act3.0-mediated T_1 *Arabidopsis* plants. The extra-early-flowering phenotype is observed in CBE-Cas9n-Act3.0-A + BE T_1 populations. Two 15-nt protospacers within gR2.0 were co-expressed for *AtFT* activation. Two 20-nt protospacers within gR1.0 were expressed for base editing of *AtALS* and *AtACC2*, respectively. Blue dashed circles indicate extra-early-flowering plants. **b**,**e**, Representative genotypes of *atalsatacc2* in CBE-Cas9n-Act3.0-A + BE T_1 populations (**b**) and T_2 T-DNA free standard-flowering populations (**e**) analyzed by using CRISPR RGEN tools. The DNA C bases within the protospacer sequence are marked in orange, and the numbers suggest the ratio of DNA base C or T in total reads. **c**, Representative results of C-to-T base editing in CBE-Cas9n-Act3.0 T_1 extra-early-, early- and standard-flowering plants. A total of 48 independent plants were examined. Box plots, 25th–75th percentile; center line, median; whiskers, full data range in **b** and **c**. Each dot indicates an individual plant. *P* values were obtained by using the two-tailed Student's *t* test. $^*P < 0.05$, $^{**}P < 0.01$. **d**, Representative phenotypes of the progenies of the T_1 extra-early-flowering plants. **f**, Representative mutation analysis of T_2 T-DNA free standard-flowering populations by using NGS. Each dot indicates an individual plant. Error bars represent the mean ± s.d. ($n = 29$, 24, 38 and 32 independent descendants for T_1 #5, #14, #16 and #17 lines, respectively). A, activation; BE, base editing. Figure adapted with permission from ref. [23], Springer Nature Limited.

Part 2: enhancing rice regeneration and heritable mutations in an HF manner

A representative example of enhancing rice regeneration and heritable mutations in an HF manner is given in Fig. 5. Using the Cas9-Act3.0 system, we demonstrated that this system enabled simultaneous high-efficiency genome editing of two target genes (*OsGW2* and *OsGN1a*) with concurrent activation of the morphogenic gene *OsBBM1* in rice plants[23] (Fig. 5). Cas9-Act3.0-induced *OsBBM1* activation led to the regeneration of transformed calli and seedlings in the HF culture medium (Fig. 5a). We also found that the heritable targeted mutations of both *OsGW2* and *OsGN1a* were significantly enriched by using the HF method (Fig. 5b,c). Overall, Combo-induced activation of *OsBBM1* enables the regeneration of genome-edited plants in an HF manner as well as enrichment of the heritable targeted mutations.

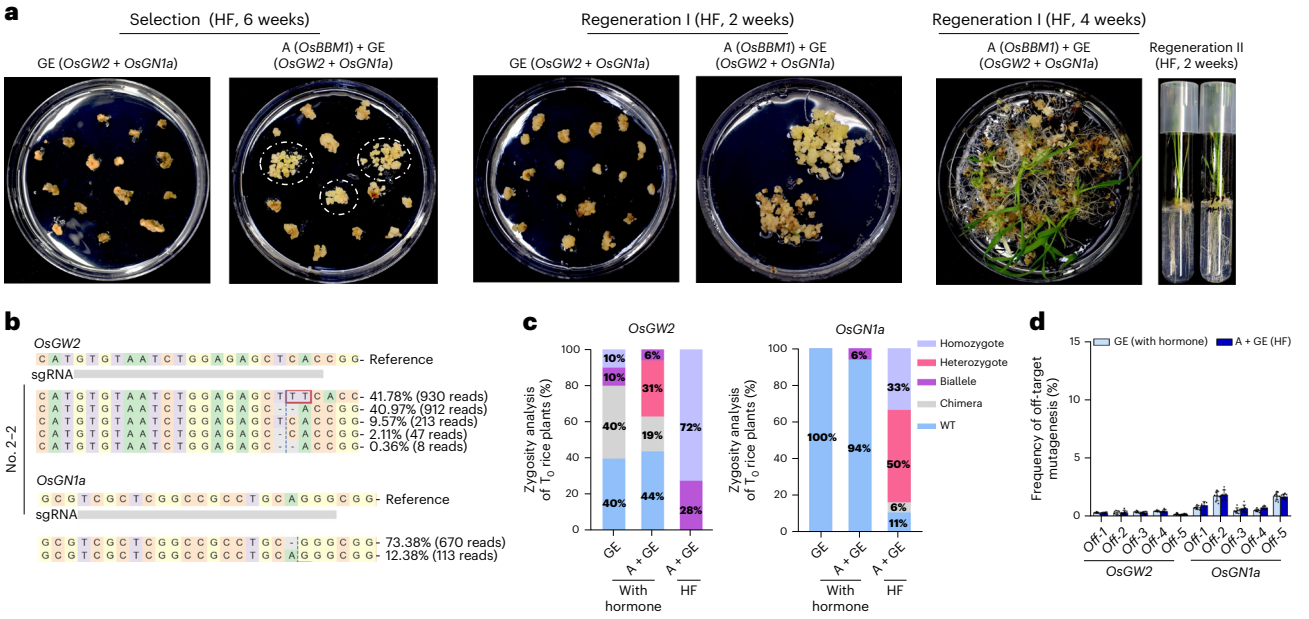

Fig. 5 | Anticipated results of Cas9-Act3.0-mediated rice regeneration and heritable mutations in a hormone-free manner via Combo-mediated activation of *OsBBM1*. **a**, Representative results of Cas9-Act3.0-mediated callus induction and regeneration in HF medium by activating *OsBMM1*. All calli were sub-cultured by using fresh medium without any hormone every 2 weeks. The white dashed circles indicate the growing calli. **b**, Representative alleles of *OsGW2* and *OsGN1a* in Cas9-Act3.0 T_0 populations analyzed by using the CRISPResso2 tool. The horizontal and vertical dashes indicate DNA base deletion and the expected cleavage site, respectively. The red box indicates the nucleotide insertion. Each row represents a unique genotype as well as its frequency. **c**, Representative zygosity analysis of Cas9-Act3.0 T_0 populations at both target sites of *OsGW2* and *OsGN1a*. The frequencies of each zygotic type are shown as percentages. The Cas9-Act3.0 plants regenerated from medium with hormones are used as the control. **d**, Representative off-target analysis by CRISPResso2 of Cas9-Act3.0 T_0 rice plants at both *OsGW2* and *OsGN1a* target sites. The Cas9-Act3.0 plants regenerated from medium with hormones are used as the control. Each dot indicates one individual plant. Error bars represent the mean ± s.d. ($n = 8$ independent plants). A+GE (HF), simultaneous gene activation and editing in an HF manner; GE, genome editing; Off-1 to Off-5, off-target sites. Figure adapted with permission from ref. [23], Springer Nature Limited.

Data availability

No new data were generated for this protocol, and all presented data in this protocol are available in the primary supporting paper[23].

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Author contributions

C.P. wrote the manuscript. Y.Q. and C.P. revised the manuscript.

Competing interests

Y.Q. and C.P. are inventors on a US patent application that has been filed on the CRISPR-Combo system in this protocol. Y.Q. is a consultant for Inari Agriculture.

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