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Guide RNA library-based CRISPR screens in plants: opportunities and challenges

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Next-generation sequencing technologies have revolutionized our ability to read sequence information at the genome and transcriptome levels in a high-throughput manner. However, genetic screening at a large or genomic scale remains challenging in plants. Recently, the RNA-guided CRISPR–Cas nucleases have been optimized for high-throughput functional genomic screens combined with guide RNA (gRNA) libraries in plants. This approach has shown great promise in facilitating genetic screening, directed evolution, and quantitative trait engineering. However, this technology is still in its infancy. In this short review, we describe the recent progress in gRNA library-based CRISPR screens in plants. We provide a critical assessment of the current approaches and emerging delivery methods for CRISPR screens. We also highlight the challenges and present future perspectives on CRISPR screens in plants.

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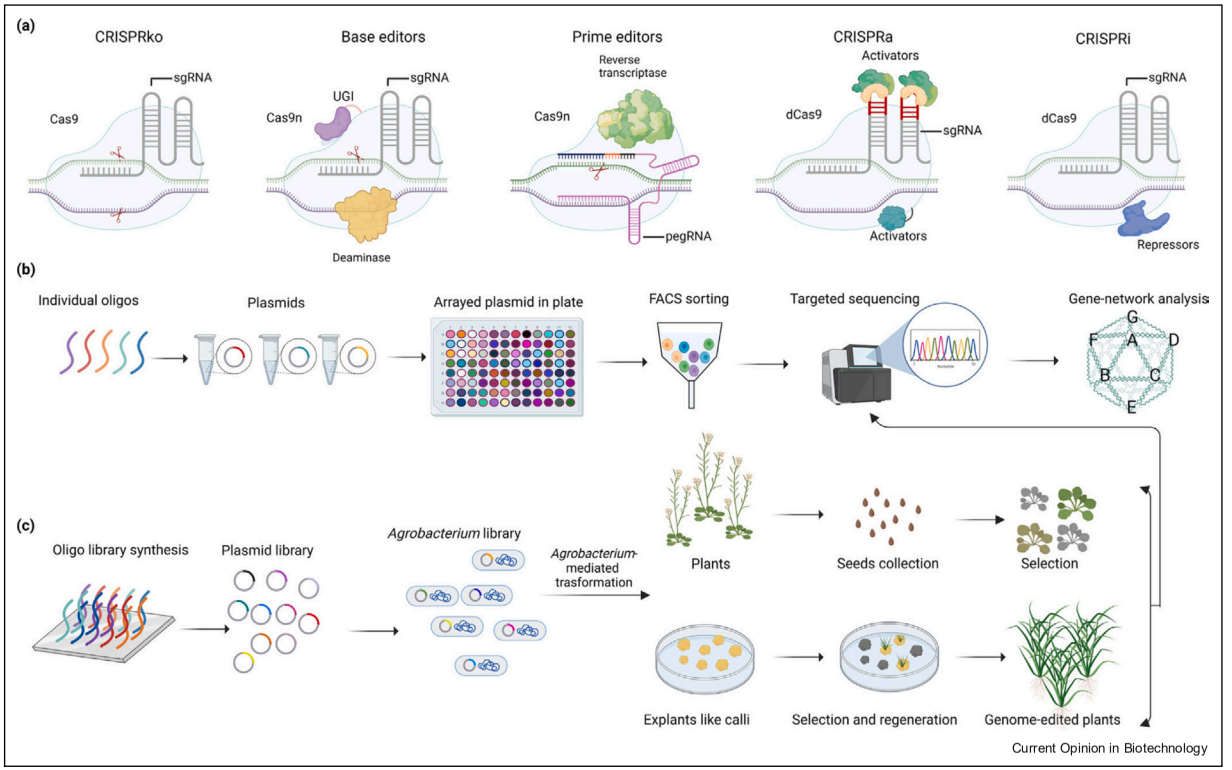
Introduction

The exponential increase of assembled plant genomes inspires the development of more effective functional genomic approaches to define gene function. Sequence-specific nucleases (SSNs), in particular, the type-II clustered regularly interspaced short palindromic repeats (CRISPR) and clustered regularly interspaced short palindromic repeats-associated protein 9 (Cas9) [1] and Cas12a (formerly Cpf1) [2] have emerged as promising genetic perturbation systems. DNA double-strand breaks (DSBs) are induced by SSNs (e.g. CRISPR/Cas9) (Figure 1a) and predominately repaired by non-homologous end-joining (NHEJ) [3]. NHEJ-introduced insertions and deletions (indels) can result in a frame-shift that likely introduces a premature stop codon. Owing to the ease of designing, gRNA targeting almost all genome loci can be quickly synthesized. Therefore, gRNA library-based CRISPR/Cas systems have been quickly adopted for high-throughput loss-of-function screens even on a genome-wide scale [4,5].

Recently, DSB-independent CRISPR-based methods such as CRISPR-mediated base editing (Figure 1a) [6,7] and prime editing (Figure 1a) [8] technologies have been developed to generate nucleotide variation in the genome. Two major classes of base editors, cytidine base editors (CBEs) [7] and adenine base editors (ABEs) [6] (Figure 1a), have been shown to introduce base conversions within a defined nucleotide window. CBEs and ABEs resulting from the fusion of nickase Cas9 (nCas9, Cas9D10A) with either the cytidine deaminase (e.g. APOBEC1, BE3) or the adenine deaminase (e.g. TadA, ABE) enable programmable C•G-to-T•A or A•T-to-G•C conversion, respectively. Prime editing is a recent genome editing technology that is composed of a Cas9H840A nickase, an engineered reverse transcriptase domain, and a prime editing guide RNA (pegRNA) [8] (Figure 1a). Prime editors (PEs) can introduce all 12 types of base-to-base conversions, and install small insertions and deletions in a precise and targeted manner without donor DNA templates.

Beyond genome editing, CRISPR/Cas systems have been engineered as a programmable RNA-guided platform for transcription regulation [9,10] (Figure 1a). A catalytically inactive Cas protein (dCas) that lacks DNA cleavage activity but remains competent for RNA-guided DNA binding is utilized in CRISPR/dCas systems for various purposes. Functional units such as transcriptional activators and repressors can be fused to dCas protein, enabling efficient CRISPR-mediated transcriptional activation (CRISPRa) and interference (CRISPRi), respectively [9] (Figure 1a).

Figure 1



Schematic representation of CRISPR systems and screening approaches. **(a)** Five classes of CRISPR-based screening systems are CRISPRko, base editors, PEs, CRISPRa, and CRISPRi. For simple depiction, only the CRISPR/Cas9 is shown, while we acknowledge that more CRISPR/Cas nucleases other than Cas9 are available. Cas9 nucleases guided by sgRNA are allowed to generate blunt-end DSBs at the target site using two distinct nuclease domains (RuvC and HNH). CBEs, depicted here, induce C•G-to-T•A point mutations using clustered regularly interspaced short palindromic repeats-associated protein 9 nickases (Cas9n) fused to cytidine deaminases and uracil glycosylase inhibitor domains (UGI). While adenine base editors (ABEs) induce A•T-to-G•C point mutations using Cas9n fused to adenine deaminases. PE consists of a Cas9n, a reverse transcriptase domain, and a pegRNA. The catalytically inactive Cas9 (dCas9) proteins can be fused with transcriptional activators or repressors, enabling efficient gene-specific CRISPRa or CRISPRi. **(b,c)** Two classes of genetic screening strategies, arrayed screens **(b)** and pooled screens **(c)**, are designed on cell and plant levels, respectively. In arrayed screens, CRISPR reagents are separately constructed and introduced into cells in multiwell plates. Screen readout represents the results of cell population in individual wells. In pooled screens, all CRISPR reagents are synthesized and constructed as a pooled library. The pooled CRISPR screening library is delivered into plants typically using the *Agrobacterium*-mediated transformation method. The transgenic lines can be traced by the identification of the unique gRNA sequences integrated into their genome using PCR (polymerase chain reaction) amplification and next-generation sequencing. Fluorescence-activated cell sorting (FACS). The figure was created with BioRender.

These new CRISPR/Cas systems have further broadened the capabilities of CRISPR screens in the life sciences. One of the most appealing features of CRISPR/Cas-mediated genetic screens is that the unique gRNAs can serve as barcodes, which enable the rapid connection between the phenotype and genotype, a central goal that has been pursued through either forward or reverse genetic screens. Here, we provide a succinct overview of the advances of CRISPR screens in plant research. We also discuss the current challenges and potential perspectives for future improvements in CRISPR screening.

Libraries and CRISPR screens strategies

Two popular CRISPR/Cas genetic screens have been conducted in previous studies [5,11], arrayed (Figure 1b), or pooled screens (Figure 1c). In the arrayed screens, individual CRISPR reagents were separately prepared and introduced into the culture well of a multiwell plate (Figure 1b). The arrayed screens are typically low throughput and labor-intensive. Another disadvantage is that the studies based on arrayed screens are mainly limited to the cellular level. These shortcomings have prevented the wide application of arrayed screens in plant research.

Compared with the arrayed screens, the CRISPR-based pooled screens have been adopted in high-throughput functional screens in plants in proof-of-concept studies [12,13]. In pooled screens, *in silico*-designed gRNAs targeting multiple genes are synthesized as a pool of oligonucleotides (gRNA library) [12–14] (Figure 1c). The gRNA sequences can serve as barcodes for the library screening readouts. These oligonucleotides are then cloned into expression vectors to generate a pooled plasmid library. In plant research, the pooled library is typically transformed into *Agrobacterium* cells and then used to create a large mutagenized population by *Agrobacterium tumefaciens*-mediated transformation [12,13]. Next, genotypes of all transgenic lines or those with the phenotypes of interest are analyzed by first sequencing across the gRNA-containing T-DNA regions to identify the corresponding single-guide RNAs (sgRNAs) and then sequencing the corresponding genomic target regions using NGS (next-generation sequencing) to figure out the genotype (Figure 1a and b). Significant enrichment of specific gRNAs indicates the causal links between the targeted genes and phenotypes of interest.

CRISPR/Cas screens for high-throughput functional genomics

Genome-scale functional genomics screening allows a high-throughput identification of the causal links between genotypes and phenotypes (Figure 2a). Two studies have successfully used CRISPR/Cas9 for genome-wide functional screens in rice (*Oryza sativa*) [12,13] (Table 1). Lu et al. generated a pooled library of 88 541 gRNAs targeting 34 234 genes [12]. A total of 84 384 T_0 transgenic lines with around 80% targeted mutagenesis frequency were generated. Phenotypic alterations in fertility, growth, tiller angle, and leaf color were observed in T_0 plants grown in the field. Similarly, Meng et al. constructed a pooled library of 25 604 gRNAs targeting 12 802 genes highly expressed in rice shoot tissue [13]. In addition, small-scale CRISPR-based functional genomics screening has been executed in tomato (*Solanum lycopersicum*) [15], soybean (*Glycine max* (L.) Merr.) [16], and maize (*Zea mays*) [17] with pooled libraries of 165, 70, and 1368 gRNAs, respectively (Table 1). More recently, Chen et al. developed a FLASH pipeline for high-throughput genetic screening of 1072 members of the receptor-like kinase family in rice using an arrayed CRISPR library [18] (Table 1).

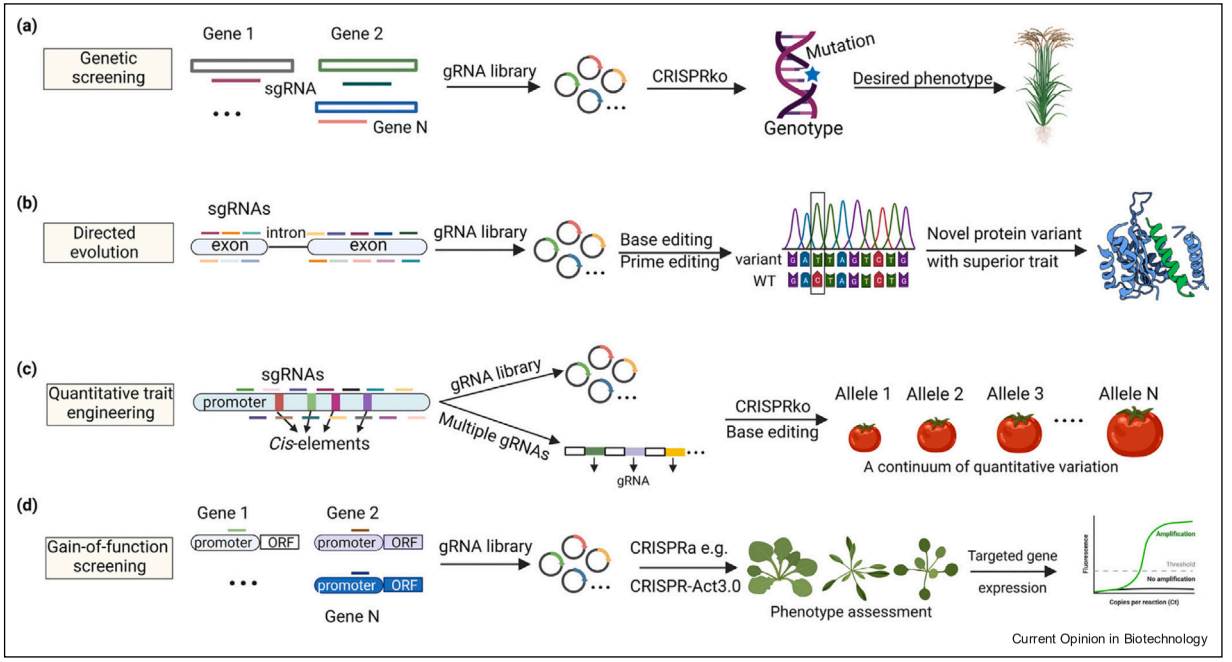
These proof-of-concept studies provide a workflow for performing CRISPR-based functional genomics screening in plants (Figure 2a), highlighting the flexibility and scalability of CRISPR screens. Unlike the conventional random mutagenesis methods, target genes can be customized in CRISPR screens, from a few gene family members to all annotated genes in a genome.

CRISPR/Cas screens for directed evolution

Genetic variants lead to genetic diversity, contributing to trait evolution and crop domestication [19]. Conventionally, natural mutations or physical and chemical mutagenesis randomly induce genetic variations. By contrast, CRISPR/Cas-based directed evolution is efficient and powerful as mutations are induced directly at the target gene, instead of randomly at the whole genome [20,21] (Figure 2b). Using clustered regularly interspaced short palindromic repeats knockout (CRISPRko) approach, Butt et al. successfully induced variants of the spliceosome component SF3B1 for resistance to splicing inhibitors [22] (Table 1). A pooled library of 119 gRNAs representing all protospacer-adjacent motif sites in the whole coding sequence of *SF3B1* was employed. Since CRISPR/Cas nucleases predominantly introduce indels rather than nucleotide substitutions, they are not most suitable for directed protein evolution.

However, CRISPR-derived base editors enable precise conversion of individual nucleotides within editing windows [23,24], making them better tools for directed evolution (Figure 2b). Combined with gRNA libraries, base

Figure 2



Applications of CRISPR screens in plants. **(a)** Genetic screening based on loss-of-function knockout (CRISPRko) where a Cas nuclease is typically used. **(b)** Directed evolution of target proteins for gain-of-function or altered function where based editors or PEs are used. **(c)** Quantitative trait engineering where cis-elements are targeted by Cas nucleases or base editors either using a gRNA library or multiplexed expression of multiple gRNAs. **(d)** Gain-of-function screening based on a CRISPRa system such as CRISPR-Act3.0 where a catalytically inactivated Cas protein is typically used. ORF, open reading frame. The figure was created with BioRender.

Table 1

Guide RNA library-based CRISPR screens in plants.

Purposes	Species	Screening approaches	Library delivery	gRNA library size	Number of targeted genes	Coverage (gRNA per gene)	Population size	Mutation efficiency	Refs
Functional genomics screening	Rice	CRISPRko	*Agrobacterium*	88,541	34,234	2.6	84,384	83.90%	[12]
	Rice	CRISPRko	*Agrobacterium*	25,604	12,802	2	14,000	63.30%	[13]
	Rice	CRISPRko	*Agrobacterium*	12 (per library) x 90	1072	1	5,039	92.10%	[18]
	Tomato	CRISPRko	*Agrobacterium*	165	54	3	31	62.50%	[15]
	Tomato	CRISPRko	*Agrobacterium*	36	18	2	59	71%	[15]
	Soybean	CRISPRko	*Agrobacterium*	70	102	1	407	59.20%	[16]
	Maize	CRISPRko	*Agrobacterium*	196	98	2	886	85%	[17]
	Maize	CRISPRko	*Agrobacterium*	1,181	1,181	1	3,470	53.70%	[17]
Directed evolution	Rice	CRISPRko	*Agrobacterium*	119	1 (*SF3B1*)	119	15,000 calli	NA (21 GEX1-resistant calli)	[22]
	Rice	CBE	*Agrobacterium*	12	1 (*OsALS*)	12	NA	NA (12 resistant calli)	[26]
	Rice	CBE	*Agrobacterium*	15	1 (*OsALS*)	15	120 calli	2.5% (3 resistant calli)	[27]
	Rice	CBE	*Agrobacterium*	8	1 (*OsEPSPS*)	8	NA	NA (2 resistant calli)	[27]
	Rice	CBE and ABE	*Agrobacterium* and particle bombardment	63	1 (*OsALS1*)	63	36,00 calli	28% (113 resistant calli)	[25]
	Rice	CBE and ABE	*Agrobacterium*	141	1 (*OsACC*)	141	5,000 calli	NA (25 resistant calli)	[28]
	Rice	STEMEs	*Agrobacterium*	200	1 (*OsACC*)	200	NA	13.18% (17 resistant calli)	[29]
	Rice	Prime editing	*Agrobacterium*	64 (per library) x 6	1 (*OsACC*)	384	1,500×6 calli	NA (377 resistant calli)	[30]
Quantitative trait engineering	Tomato	CRISPRko	*Agrobacterium*	8	1 (*SlCLV3*)	8	NA	NA (30 *slclv3*^{pro} alleles)	[32,33]
	Tomato	CRISPRko	*Agrobacterium*	8	1 (*SlWUS*)	8	NA	NA (8 *slwus*^{pro} alleles)	[33]
	Tomato	CRISPRko	*Agrobacterium*	8	1 (*SlWOX9*)	8	NA	NA (23 *slwox9*^{pro} alleles)	[35]
	Maize	CRISPRko	*Agrobacterium*	9	1 (*ZmCLE7*)	9	NA	NA (6 *zmcle7*^{pro} alleles)	[34]
	Maize	CRISPRko	*Agrobacterium*	9	1 (*ZmFCP1*)	9	NA	NA (5 *zmfcp1*^{pro} alleles)	[34]
	Rice	CRISPRko	*Agrobacterium*	39	1 (*IPA1*)	39	792	62.40%	[36]

editors allow high-throughput protein variant screening at endogenous locus. Several groups have applied these tools to the directed evolution of herbicide-tolerance genes such as *ACETOLACTATE SYNTHASE1* (*OsALS1*) [25–27], *5-ENOLPYRUVYLSHIKIMATE-3-PHOSPHATE SYNTHASE* (*EPSPS)* [27], and *ACETYL-COENZYME A CARBOXYLASE* (*OsACC*) [28,29] (Table 1). Either CBEs or ABEs, when applied in isolation, can only confer one type of base transition that is C-to-T or A-to-G. To diversify the base editing outcomes, Li et al. developed a dual cytosine and ABE system with a demonstration of near-saturation mutagenesis of the carboxyltransferase domain of *OsACC* using a pooled library of 200 gRNAs [29] (Table 1).

To confer targeted saturation mutagenesis, one could use PEs. A recent study applied PE-based library screening to the directed evolution of *OsACC* in rice [30] using a total of 64 pegRNAs conferring all possible amino acid changes of a targeted three-nucleotide unit (Table 1). These reports indicate that base editors and PEs would greatly facilitate direct evolution and trait improvement for agriculture (Figure 2b).

CRISPR/Cas screens for quantitative trait engineering

Noncoding *cis*-regulatory elements control gene expression and play a central role in the evolution of quantitative traits [31]. CRISPR/Cas screens can generate diverse *cis*-regulatory alleles in a high-throughput manner, contributing to crop domestication and quantitative trait engineering (Figure 2c). Wang et al. employed CRISPR/Cas9 with eight sgRNAs targeting the *cis*-regulatory sequences of *SlCLV3* (*CLAVATA3*) and *SlWUS (WUSCHEL)* promoters, respectively, generating a range of quantitative variations of stem cell proliferation and fruit size in tomato [32,33] (Table 1) (Figure 2c). Similarly, Liu et al. used CRISPR/Cas9 with nine sgRNAs to edit the *cis*-regulatory regions of *CLE* (*CLAVATA3/ESR-RELATED*) promoter in maize, resulting in weak promoter alleles of *CLE* and enhanced grain-yield-related traits [34] (Table 1). By screening the *cis*-regulatory regions of *WOX9* (*WUSCHEL HOMEOBOX9*) promoter using CRISPR/Cas9 with eight sgRNAs, the Lippman's group further revealed the hidden pleiotropic roles of *WOX9* in vegetative and reproductive development in tomato [35] (Table 1). Comparing with the canonical sgRNA library-based CRISPR screens, these studies represent a new kind of CRISPR screen since multiplexed sgRNA expression was used to generate a library of promoter editing events with large deletions and inversions [32–35] (Figure 2c). By contrast, in rice, one group utilized a tiling-deletion-based screen for the *cis*-regulatory regions of *IPA1* (IPA1-interacting protein 1) via CRISPR/Cas9 with a pooled library of 39 sgRNAs [36] (Table 1). They identified one allele with a certain *cis*-regulatory region deletion that can increase both panicle number and size, leading to enhanced rice yield [36]. These studies, despite with limited scope and resolution, have demonstrated CRISPR/Cas-based screens on *cis*-regulatory elements as a promising approach for rapidly developing quantitative traits in crop domestication and breeding.

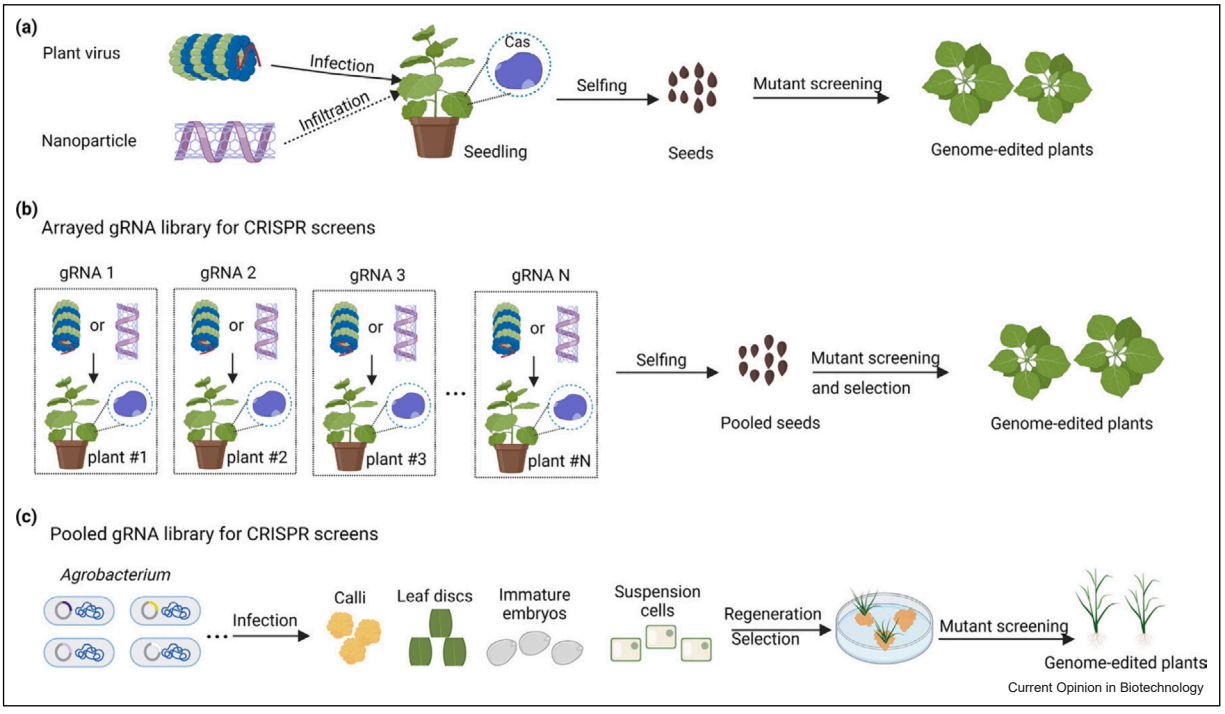
Agrobacterium-mediated delivery is the most promising method for pooled clustered regularly interspaced short palindromic repeats screens in plants

An effective and universal method of delivering CRISPR reagents is essential for the high-throughput application of CRISPR–Cas screens in plants. For delivering the pooled CRISPR libraries in plants, only *Agrobacterium tumefaciens*-mediated transformation has been demonstrated in previous reports [5] (Table 1) (Figure 1c). This is because an individual cell/plant receives only one T-DNA integration from *A. tumefaciens* most times, which allows for establishing a simple linkage between genotype and phenotype (Figure 2a). By contrast, nonbiotic delivery methods such as polyethylene glycol and biolistic delivery are not suitable for large-scale gRNA library-based screens unless they provide a solution to meet the one or few gRNAs per cell requirement. However, *Agrobacterium*-mediated transformation is limited to a narrow range of plant species, and it may not work for all genotypes with one plant species [37]. Developing efficient *Agrobacterium*-mediated transformation protocols for recalcitrant plant species or genotypes is needed. On the other hand, *Agrobacterium*-mediated stable transformation generally takes a long period for the whole plant regeneration from the transformed tissues or cells [37]. Therefore, new techniques and approaches for accelerating or avoiding tissue culture are needed before CRISPR screens to reach their full potential. Encouraging progresses have been reported on co-expression of plant growth regulators [38,39] or direct activation of the endogenous growth regulators using CRISPR-Combo that enables orthogonal gene activation and editing [40].

Virus-mediated delivery can be used for arrayed CRISPR screens

To overcome the tissue culture bottleneck, plant virus-induced genome editing technologies have been developed, enabling delivering CRISPR reagents to generate germline mutations (Figure 3a). Ellison et al. first successfully generated efficient multiplexed heritable gene editing using an engineered *Tobacco rattle virus* (TRV) via increasing the mobility of gRNAs by fusing gRNA with a truncated *flowering Locus T* (FT) in *Nicotiana benthamiana* [41]. Following this, a fusion of tRNA isoleukine ($tRNA^{Ileu}$) to sgRNA has been shown to induce highly efficient heritable gene knockout and base

Figure 3



Representative CRISPR reagent delivery in plants. **(a)** Plant virus- and nanoparticle-mediated delivery of CRISPR reagents into intact plants. Dash line indicates that nanoparticle-based CRISPR reagent delivery has not been demonstrated yet. **(b)** Plant virus and nanoparticle could be used for arrayed CRISPR reagents in plants. **(c)** *Agrobacterium*-mediated delivery represents the most promising method for pooled CRISPR screens. The figure was created with BioRender.

editing outcomes in *Arabidopsis* using TRV delivery [42,43]. Besides dicots, the other two studies achieved up to 100% heritable editing in monocot wheat using *Barely stripe mosaic virus* (BSMV) [44,45]. All these studies used positive-strand RNA viruses to deliver CRISPR gRNAs to transgenic plants expressing Cas9 or CBE (Figure 3a). Therefore, there are still limitations to employing positive-strand RNA viruses such as TRV and BSMV to achieve germline mutations in plants without demonstrated stable transformation procedures.

Ma et al. recently successfully delivered the entire CRISPR/Cas9 components into *Nicotiana benthamiana* and achieved highly efficient DNA-free genome editing by using Sonchus yellow net rhabdovirus (SYNV), a negative-strand RNA virus [46]. SYNV has a large cargo capacity and ensures the success of delivering the whole CRISPR/Cas9 system in plant cells, which cannot be achieved in most DNA viruses or positive-strand RNA viruses with limited capacity for delivering DNA/RNA fragments [46]. It is worth noting that SYNV-mediated DNA-free germline editing is still dependent on tissue culture regenerated plants from virus-infected tissues [46].

The virus-based delivery tools are still limited by tissue culture at a certain level, however, promise to make them perform germline editing for arrayed CRISPR screens in plants (Figure 3b). In the future, technological advances on viruses that prevent co-infection or subsequent infection in host cells will pave the way toward pooled gRNA library-based CRISPR screens in plants.

Nanoparticle-mediated delivery may be used for arrayed CRISPR screens

Recent breakthroughs in nanomaterial synthesis have enabled nanoparticles to deliver DNA/RNA reagents to intact plants for genetic engineering applications [47–49] (Figure 3a). Compared with conventional delivery methods, nanomaterials enable the delivery of functional biomolecules to previously inaccessible plant tissues and organelles in a DNA integration-free and species-independent manner [47–49]. Although nanoparticle-mediated DNA/RNA delivery has been applied in a wide range of plant species, including cotton, tobacco, arugula, wheat, *Arabidopsis*, and maize [50,51], nanomaterial-mediated CRISPR reagent delivery in plants has not yet been reported. The major challenges are the large size and unique physicochemical properties of the CRISPR/Cas complex where attaching Cas protein with nanomaterial is not stable or efficient [50]. There are clear barriers hindering nanoparticle delivery for high-throughput CRISPR screens. By overcoming these

barriers, nanoparticles could play a critical role in facilitating arrayed CRISPR screens to realize their full potential in plant genetic engineering (Figure 3b).

Conclusions and perspectives

High-throughput CRISPR screens as a game-changing approach could potentially revolutionize basic and applied research in plants. Four classes of CRISPR–Cas-derived screening strategies, including targeted gene knockout [12,13,15–17,22], base editing [25–27], prime editing [30], and transcriptional regulation [9], are currently available for genome-scale screening. They are typically complementary, thus, the combinatorial CRISPR screens [40] further offer an opportunity to comprehensively understand plant functional genomics. With the robust CRISPR-Act3.0 gene activation system, gain-of-function CRISPR screen should soon be realized in plants (Figure 2d). Furthermore, the combination of single-cell sequencing with CRISPR screens allows for high-resolution characterization of the causal gene regulatory network at the single-cell level [52]. Currently, the *Agrobacterium*-mediated delivery represents the most promising method for gRNA library-based CRISPR screens in plants (Figure 3c). However, each strategy comes with its inherent capabilities and limitations. Further improvements in development of species-independent CRISPR reagent delivery methods and improving their editing capabilities are most critical to achieving the full potential of CRISPR screens. Taken together, the gRNA library-based CRISPR screening is leading us into a new era, and the continued efforts to improve these CRISPR screening approaches will undoubtedly benefit crop improvement and future agriculture.

CRediT authorship contribution statement

C.P. wrote the draft of the review. C.P. prepared the table and figures displayed in the review. Y.Q. provided Supervision. Y.Q., G.L., and A.B. revised the paper. All authors read and approved the final version of the paper.

Conflict of interest statement

Y.Q. is a consultant for Inari Agriculture and CTC Genomics. A. B. is an employee of Reliance Industries Limited. The remaining authors declare no competing interests.

Data Availability

No data were used for the research described in the article.

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- • of special interest
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