1 REVIEW

2 CRISPR Screens in Plants: Approaches, Guidelines and Future Prospects

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13 Short Title: Review of CRISPR screens in plants

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15 One-sentence summary: Recent advances, methodologies and future prospects for CRISPR
16 screens in plants.

18 Abstract

19 CRISPR-Cas systems have revolutionized genome engineering by facilitating a wide range of
20 targeted DNA perturbations. These systems have resulted in new powerful screens to test
21 gene functions at the genomic scale. While there is tremendous potential for CRISPR screens
22 to map and interrogate gene regulatory networks at unprecedented speed and scale, their
23 implementation in plants remains in its infancy. Here we discuss the general concepts, tools
24 and workflows for establishing CRISPR screens in plants and analyze the handful of recent
25 reports using this strategy to generate mutant knockout collections or diversify DNA
26 sequences. In addition, we provide insight on how to design CRISPR knockout screens in
27 plants given the current challenges and limitations and examine multiple design options.
28 Finally, we discuss the unique multiplexing capabilities of CRISPR screens to investigate
29 redundant gene function in highly duplicated plant genomes. Combinatorial mutant screens
30 have the potential to routinely generate higher-order mutant collections and facilitate the
31 characterization of gene networks. By integrating this approach with the large resource of
32 genomic profiles that were generated in the last two decades, the implementation of CRISPR
33 screens offers new opportunities to analyze plant genomes at deeper resolution and will
34 greatly advance plant functional and synthetic biology.

36 INTRODUCTION

37 The emergence of genome editing tools has offered great opportunities for deciphering gene
38 function as well as engineering beneficial traits in a wide range of biological systems. Among
39 these tools, the clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-
40 associated nuclease (Cas) system has been harnessed to selectively introduce mutations
41 across life kingdoms (Knott and Doudna, 2018). The simplicity and modularity of CRISPR
42 systems have led to a great expansion of the genetic toolbox as well as a diversification of its
43 applications in various organisms (Knott and Doudna, 2018). In particular, the building of large
44 CRISPR libraries has driven the implementation of forward genetic screens in which
45 mutations are permanently introduced at the genome-scale with high precision (Smith et al.,
46 2017). These recent improvements in screening methodologies have primarily been used for
47 functional genomics as well as for drug target discovery in mammalian cell systems (Behan et
48 al., 2019; Shalem et al., 2015). Furthermore, saturation mutagenesis has enabled high-
49 throughput protein engineering (Kweon et al., 2020; Li et al., 2020; Ma et al., 2017).

50 Besides its applications in animal systems, the CRISPR-Cas system has been optimized for
51 plant genome editing and a large toolbox is now available to engineer many plant species and
52 crops (Manghwar et al., 2019; Zhang et al., 2019b). In contrast to small-scale gene knockout
53 experiments, the application of high-throughput screening in plants is still in its infancy
54 compared to animal cell culture systems. We anticipate that CRISPR screens will become a
55 widely-used functional genomics tool for plant research in the coming years and will
56 significantly expand our understanding of plant gene function as well as facilitate the
57 engineering of crop traits. In this review, we discuss the principles and methods for building
58 high-throughput CRISPR screening platforms as well as the specific challenges and
59 advantages when implementing this technology in plants. We describe current studies that
60 have used this approach for building plant knockout mutant collections or engineering and
61 evolving new genetic variants. Finally, we discuss the future potential of CRISPR screening
62 and its applications in various plant species.

63 CRISPR-CAS GENOME EDITING TOOLS

64 At the molecular level, the CRISPR-Cas system is composed of two core components that
65 form a ribonucleic complex: the CRISPR-associated9 (Cas9) nuclease and the guide RNA
66 (gRNA) (Figure 1). The gRNA is a chimeric molecule composed of the tracrRNA, which is

67 required for binding Cas9, and the crRNA, which is a 20-nt sequence complementary to the
68 target DNA (protospacer) (Deltcheva et al., 2011; Jiang and Doudna, 2017; Jinek et al., 2012).
69 Base pairing of the gRNA with DNA forms an RNA-DNA heteroduplex, which promotes Cas9-
70 mediated cleavage of double-stranded DNA through the activity of two catalytic domains: the
71 HNH and RuvC cutting the target and the non-target strands, respectively (Jinek et al., 2012;
72 Nishimasu et al., 2018). The *Streptococcus pyogenes* Cas9 (SpCas9) binds DNA at NGG
73 protospacer adjacent motif (PAM) sequences and typically introduces a blunt, DNA double
74 strand break (DSB) between the 3rd and 4th nucleotides away from the PAM (Figure 1; (Jinek
75 et al., 2012; Sternberg et al., 2014)). In a cell, the endogenous DNA repair machinery is then
76 activated to repair DSBs and imperfect repair introduces insertions or deletions (indels) at the
77 break site. When these indels appear in coding sequences, frameshifts or premature stop
78 codons can disrupt the expression of the gene product whereas deletions in regulatory
79 regions can significantly alter gene expression (Canver et al., 2015; Cong et al., 2013; Jinek
80 et al., 2012; Rodríguez-Leal et al., 2017). The DNA recognition capabilities and nuclease
81 activity of Cas enzymes can be uncoupled by inactivating the two catalytic domains of Cas9
82 through two amino acid substitutions (D10A, H840A) (Jinek et al., 2012). The resulting Cas9,
83 referred to as dCas9, binds target DNA without generating a DNA break and has been used
84 as a platform to recruit enzymes or protein tags at specific genomic sites and introduce
85 modifications such as point mutations, changes in transcriptional response or chromatin
86 accessibility (Deng et al., 2015; Gilbert et al., 2013; Komor et al., 2016; Liu et al., 2016).

87 Base editors have been designed by fusing dCas9 or nickase Cas9 (Cas9(D10A); nCas9)
88 (Figure 1B), with cytidine deaminases, catalyzing cytidine (C) to thymine (T) nucleotide
89 transitions (Komor et al., 2016). Upon binding the complex to the target DNA, Cs on the non-
90 target strand are deaminated into uracil (U) (Komor et al., 2016). After preferential repair of
91 the non-edited strand by the DNA repair machinery, a stable transition from C to T is
92 introduced. Additionally, the C to guanine (G) transversion can also result from incorrect
93 repair (Hess et al., 2016; Komor et al., 2016). Along the same lines, adenine (A) base editors
94 mutate A into G by deaminating A into inosine (I), which is then recognized by the cellular
95 repair machinery as G (Gaudelli et al., 2017). Base editors operate under a restricted editing
96 window on the DNA and the choice and combination of nuclease variants with deaminases
97 have an impact on which nucleotides are targeted (Rees and Liu, 2018). For example, the
98 cytidine deaminase base editor BE3, which uses nCas9 fused to APOBEC1, shows strong

99 activity on nucleotides +4 to +8 of the protospacer (Komor et al., 2016). By contrast, CRISPR-
100 X uses a two-component system with dCas9 and a modified gRNA-recruiting AIDΔ
101 deaminase and can introduce point mutations within a window range of 100 bp centered
102 around the PAM (Hess et al., 2016). Therefore, the choice of the base editor can be tailored
103 for precise or broad editing windows.

104 In addition to the introduction of stable genetic mutations, dCas9 can also be used to
105 modulate the transcription of specific genes (Figure 1C). The CRISPRa system uses
106 transcriptional activators while CRISPRi utilizes repressors (Gilbert et al., 2013).
107 Transcriptional activators are designed by fusing dCas9 with a transactivation domains such
108 as the VP64 (Gilbert et al., 2013; Konermann et al., 2015; Lowder et al., 2018), p65 or Rta
109 domains (Chavez et al., 2015), that can be used in tandem or in repeats to increase
110 efficiency. The CRISPRa system has been demonstrated in plants and provides a
111 complementary strategy for investigating gene function by enhancing gene expression (Li et
112 al., 2017; Lowder et al., 2018). On the other hand, a repressor domain such as KRAB (Gilbert
113 et al., 2013) or the plant SRDX (Piatek et al., 2015; Tang et al., 2017) fused to dCas9 can
114 efficiently suppress expression of the targeted genes.

115 **COMPARATIVE APPROACHES FOR HIGH-THROUGHPUT SCREENING**

116 Functional genetics aims to understand gene function by characterizing how genes interact
117 with the environment and each other to result in phenotypes. Forward genetic screens are
118 powerful tools to decipher gene function without *a priori* knowledge. This approach starts with
119 a population containing genetic variation (natural or mutagenized) followed by selection of
120 individuals with phenotypes deviating from the wild-type (Alonso-Blanco et al., 2016;
121 McCouch et al., 2016; Weigel and Glazebrook, 2006). Genes are then associated with the
122 phenotypes using a variety of genetic mapping techniques and tools (Jander et al., 2002;
123 Ogura and Busch, 2016; Schneeberger et al., 2009). This approach has been used
124 successfully for several decades across all fields of biology. Building and characterizing large
125 mutagenized populations is critical for success. This has largely been done in plants by
126 randomly generating point mutations with physico-chemical treatments, transforming plant
127 populations with T-DNA or transposon systems or gene silencing (Table 1; Alonso et al.,
128 2003; Hauser et al., 2013; Kolesnik et al., 2004; Lu et al., 2018; Wang et al., 2013).

129 Chemical mutagenesis in plants largely uses ethyl methanesulfonate (EMS) that induces

multiple mutations (primarily C to T transitions) to create M₁ plant populations (Table1). It has been estimated that ~50,000 M₁ lines are required for saturation mutagenesis at all G:C bases in the *Arabidopsis* genome and this number can vary depending on genome size and mutation rate (Jander et al., 2003, Henry et al., 2014). Mutagenesis in forward genetic screens can nevertheless be used at sub-saturation levels to identify several mutant lines per gene from a relatively small population (<5,000 M₁ plants for *Arabidopsis*) (Henry et al., 2014; Lu et al., 2018; Østergaard and Yanofsky, 2004; Page and Grossniklaus, 2002; Tsai et al., 2013). As the mutant lines contain multiple mutations in addition to the causal mutation, it is necessary to perform several backcrosses, selecting only the progeny carrying the desired phenotype. The technical simplicity of this method allows it to be used on many plant species with limited genome sequence information and it has been successfully implemented to identify mutations that enhance or suppress mutant plant phenotypes (Page and Grossniklaus, 2002). Because DNA sequence changes produced by this method are not targeted to specific regions of the genome, the identification of causal mutations requires genetic mapping. The line of interest is crossed with a plant from a different genetic background to obtain a mapping population in the progeny. By using genetic markers and massively parallel sequencing-based mapping, the causal genetic region for the phenotype can then be efficiently identified (Jander et al., 2002; Page and Grossniklaus, 2002; Schneeberger et al., 2009). However, mapping causal mutations using this strategy can be time consuming especially when analyzing plant species with long generation times (Watson et al., 2018).

In contrast to chemical mutagenesis, plant populations have also been mutagenized by inserting *Agrobacterium tumefaciens* T-DNA carrying herbicide or antibiotic resistance genes or vectors carrying Ac/Ds or Mu transposons and a transposase (Kolesnik et al., 2004; May et al., 2003). Generally, mutants generated with T-DNAs or transposons contain few insertions and thus the establishment of a near saturation population using this approach requires larger populations than with EMS mutagenesis (Table 1; Østergaard and Yanofsky, 2004). One main advantage of this approach is the relative ease for causal gene identification. As the T-DNA or transposon sequence is known, insertion sites can be identified using thermal asymmetric interlaced (TAIL) PCR or other sequencing methods that do not require the establishment of a mapping population (Alonso et al., 2003; Williams-Carrier et al., 2010).

161 One limitation of EMS screening and insertion mutagenesis methods is their low capacity to
162 simultaneously target multiple genes of the same pathway in a single mutant line and thus
163 their inability to identify functionally redundant genes (Henry et al., 2014; Lu et al., 2018; Tsai
164 et al., 2013). By contrast, RNA interference and artificial microRNA (amiRNA) systems can
165 silence multiple genes by targeting conserved regions, thereby allowing the knockdown of
166 potentially functionally redundant genes (Mlotshwa et al., 2002; Schwab et al., 2006).
167 Genome-scale RNAi and amiRNA collections have been built and can be used for forward
168 genetic screening with the possibility to identify redundant genes (Hauser et al., 2013; Zhang
169 et al., 2018). However, these strategies generally result in incomplete knockout phenotypes
170 as some protein is still produced and are prone to off-targets (Echeverri et al., 2006; Jackson
171 et al., 2006).

172 In recent years, the programmable nature of the CRISPR-Cas system has enabled building
173 large knockout mutant libraries for forward genetic screens. The main advantage of using
174 CRISPR for screening lies in its ease of identifying causal genes and relatively high specificity
175 (Table 1; Liu et al., 2020; Lu et al., 2017; Meng et al., 2017). Furthermore, the possibility of
176 multiplexing allows the simultaneous targeting of multiple genes and can thus overcome
177 genetic redundancy as well as enable saturation mutagenesis with much smaller populations.
178 Finally, the versatility of the CRISPR toolbox enables generating not only indels and loss of
179 function mutations but also a range of genome perturbations such as specific single
180 nucleotide variants or transcriptional changes that are not possible with other mutagenic or
181 knockdown technologies (Gaudelli et al., 2017; Gilbert et al., 2013; Komor et al., 2016).

182

183 **APPLICATIONS OF CRISPR SCREENING IN PLANTS**

184 **Generation of Mutant Collections**

185 In contrast to animal cell systems in which CRISPR screens are widely adopted for forward
186 genetic studies, only a few have been reported in plants (Table 2). Currently the most
187 extensive pooled CRISPR mutant collections have been reported in rice using T-DNAs
188 containing a single gRNA per vector. Lu et al. targeted 34,234 non-transposable element (TE)
189 protein coding genes (~83% of all genes) of the rice genome in variety Zhonghua 11 using a
190 library of 88,541 gRNAs and two to three gRNAs per gene. They generated a T₀ mutant
191 population of 91,004 individuals, corresponding to a 1X gRNA coverage, with a mutation rate

192 approximating 80% (Lu et al., 2017). In parallel, another rice CRISPR mutant collection in the
193 same variety was created with approximately 14,000 T₀ mutant plants. The library targeted
194 12,802 highly-expressed genes in shoot tissues using two gRNAs per gene (Meng et al.,
195 2017). Based on their sequence assessment of 139 plants, the mutation rate was 63.3% with
196 33.1% homozygous, 10.8% biallelic and 19.4% heterozygous in T₀ plants, confirming the
197 efficiency and specificity of their gRNAs. Both groups identified lines with phenotypes and
198 were able to associate them with previously characterized genes. Novel phenotypes were
199 also observed and candidate genes could be identified from gRNA and target site analyses.

200 At the same time a CRISPR library was developed in tomato to investigate the function of the
201 leucine-rich repeat receptor-like kinase subfamily XII (LRR-XII) genes (Jacobs et al., 2017).
202 Fifty-four subfamily members were targeted in a pooled transformation using three gRNAs per
203 gene and 31 T₀ plants were recovered with a mutation rate of 62.5%. Nearly half of all
204 subfamily members were mutagenized with a single transformation. To increase throughput
205 and overcome potential redundancy within gene families, multiplexing CRISPR vectors were
206 then used to target 18 genes encoding putative transporters identified from differential
207 expression analysis. Using combinatorial vector assembly with three gRNAs per vector and
208 pooled plant transformation, T₀ plants were recovered with up to five mutated genes, thus
209 demonstrating the potential of generating higher-order mutant plants by combinatorial
210 targeting (Jacobs et al., 2017). Fourteen of the 18 genes were mutated at least three times,
211 demonstrating that higher replication was possible using the multiplex approach. This
212 replication made it possible to associate two developmental phenotypes with the underlying
213 mutated genes.

214 Multiplex mutagenesis can also be achieved by designing gRNAs that target homologous
215 regions of paralogous genes. Such a CRISPR library was created in soybean to target
216 duplicated genes (Bai et al., 2019). The authors observed a 60% editing efficiency and
217 generated double and triple homozygous mutant lines at the T₁ generation, which displayed
218 impaired nodule number (Bai et al., 2019). As many plant species, notably many of the
219 important crops, have undergone extensive gene and/or genome duplication, this study
220 highlights the potential of using CRISPR libraries to overcome genetic redundancy in plants.

221 Another CRISPR mutant collection was recently created in maize (Table 2; Liu et al., 2020). A
222 set of 1,244 candidate genes were selected by QTL analysis or comparative genomics. The
223 design of gRNAs for CRISPR screens requires previous knowledge of genome sequences.

224 As the authors used the non-reference KN5585 variety they first obtained the gene coding
225 sequences using *de novo* genome assembly. They then designed 1,368 gRNAs and
226 individually cloned them in a vector to target single genes. 4,356 T₀ plants were isolated and
227 subsampling showed that the mutation rate was around 85% (449/531 plants with mutation).
228 From 119 T₁ families analyzed, they isolated 107 plants showing developmental phenotypes
229 in the field (such as flowering time, plant height or architecture) and identified the putative
230 causal genes (Liu et al., 2020). Many of these confirmed previously known genes as well as
231 identified novel mutants with interesting phenotypes. This work provides a model for
232 performing a CRISPR screen on cultivars or species with limited public genomic information.

233 **Targeted Sequence Diversification**

234 The engineering of new genetic variants aims at diversifying gene sequences and creating
235 allelic series to identify proteins with novel functions. Traditionally, this has been conducted in
236 plants using two different approaches based on random mutagenesis: Targeting Induced
237 Local Lesions in Genomes (TILLING) and error-prone PCR. The TILLING method
238 mutagenizes plants by treatment with chemicals (e.g. EMS) or irradiation and has been
239 applied to many crops including wheat, rice and tomato (Jacob et al., 2018). The first
240 generation (M₁) is self-fertilized and propagated to create individual M₂ lines. DNA is isolated
241 from multiple siblings of individual M₂ lines and pooled. Gene-specific PCR amplification and
242 massive parallel sequencing can then be used to identify genetic variants in the desired gene
243 or region of interest and mutations traced back to M₂ lines for further characterization. As
244 such, TILLING collections can be used as a resource to repeatedly screen for mutant genes
245 and phenotypes. The TILLING approach has been widely applied to crops to identify genetic
246 variants underlying phenotypic traits such as pathogen resistance or pod-shattering
247 (Acevedo-Garcia et al., 2017; Jacob et al., 2018; Stephenson et al., 2019). Genetic variants
248 can also be created by error-prone PCR followed by genetic transformation. The gene of
249 interest is amplified under suboptimal buffer conditions with a DNA polymerase lacking proof
250 reading activity to promote the introduction of single nucleotide variants. The resulting gene
251 variants are then transformed into plants to test their activity (McCullum et al., 2010). This
252 method has been used to engineer broad-spectrum resistance in *R*-genes such as the *NB-*
253 *LRR Rx* gene that confers pathogen resistance in potato or to further characterize protein
254 structure-function (Farnham and Baulcombe, 2006; Harris et al., 2013).

255 In contrast to the random mutagenesis of these two approaches, CRISPR-Cas allows the
256 user to specifically target gene sequences and can encourage certain repair outcomes *in*
257 *vivo*. Rodríguez-Leal et al. made use of this by targeting the regulatory regions of the tomato
258 *CLAVATA3 (SlCLV3)* promoter using Cas9. *SlCLV3* expression controls locule number and
259 fruit size in tomato (Rodríguez-Leal et al., 2017; Xu et al., 2015). Using eight gRNAs targeting
260 a 2-kb region, they generated multiple T₀ lines carrying deletions and subsequently used one
261 of them as a parent for backcrossing with wild type. They obtained a mutant collection of
262 1,152 F₁ plants and took advantage of continued CRISPR activity on the wild-type DNA to
263 create an allelic series within the *SlCLV3* promoter. This led to plants showing a continuum of
264 variation in the number of fruit locules (Rodríguez-Leal et al., 2017). Remarkably, they next
265 used this editing strategy to engineer plant architecture variation by targeting the
266 *COMPOUND INFLORESCENCE (S)* and *SELF PRUNING (SP)* promoter using a smaller
267 segregating population of 326 and 81 F₁ plants, showing that their strategy can be applicable
268 to a number of genes to fine-tune quantitative trait variation (Rodríguez-Leal et al., 2017).

269 Along a similar line, Butt et al. used Cas9-generated indels to evolve the spliceosome and
270 identify variants that are resistant to splicing inhibitors in rice (Butt et al., 2019). They
271 transformed 119 gRNAs targeting all possible sites on the *SPLICING FACTOR 3B1 (SF3BI)*
272 coding sequence into 15,000 rice calli and applied negative selective pressure with the
273 splicing inhibitor GEX1A during regeneration. As knockout deletions of *SF3BI* are lethal, only
274 in-frame or heterozygous mutants were recovered. From this scheme, they recovered 21
275 resistant rice shoots and identified three amino acid deletions that conferred resistance to
276 GEX1A (Butt et al., 2019). They were also able to recover three additional alleles with only
277 3,000 rice calli by using a focused approach targeting known drug-interacting domains with
278 four gRNAs.

279 Another emerging strategy for sequence diversification makes use of base editors. A cytidine
280 base editor (CBE) system based on the hyperactive AID deaminase (Hess et al., 2016) and
281 an adenine base editor (ABE) were used on separate vectors to target the rice
282 *ACETOLACTATE SYNTHASE 1 (OsALS1)* gene to generate genetic variants that confer
283 resistance to the herbicide bispyribac-sodium (BS) (Kuang et al., 2020). The authors
284 constructed a library of 63 gRNAs targeting the entire *OsALS1* coding sequence and
285 observed substitutions in 20% of T₀ plants. Interestingly, editing was observed in a window
286 broader than the gRNA targeting region, suggesting that this system can be used to generate

287 mutations more broadly on the target sequence (Kuang et al., 2020). After applying negative
288 selection with BS on ~3,600 calli, they recovered 113 resistant calli and identified six *OsALS1*
289 variants conferring resistance. One new variant (P171F) was never selected in previous rice
290 breeding programs but corresponds to amino acid substitutions in a structural motif previously
291 identified in *Arabidopsis* and wheat. Using the same gRNAs and base editors, they introduced
292 the P171F substitution in the elite commercial cultivar Nangeng 46 and obtained herbicide
293 resistance, demonstrating the ability of this strategy for fast identification of genetic variants
294 and its relevance for targeted crop breeding (Kuang et al., 2020).

295 In parallel to this report, Li et al developed a dual base editor system to simultaneously target
296 genomic regions with cytidine and adenine deaminases (Li et al., 2020). The system is
297 composed of a base editor that carries both deaminases in combination with nCas9 or the
298 variant nCas9-NG, which recognizes NG, GAA and GAT PAM sites (Hu et al., 2018;
299 Nishimasu et al., 2018). This greatly broadens the number of genomic sites that can be
300 targeted by generating simultaneous A to G and G to T mutations (Li et al., 2020). The
301 authors targeted the ~1.2-kb CT domain of the rice *ACETYL-COENZYME A CARBOXYLASE*
302 (*OsACC*) which is the target of ACC herbicides. Two hundred gRNAs were subdivided into 27
303 subpools and delivered to ~6,500 rice calli. They observed an average editing efficiency of
304 13%, with 53% of the amino acids altered. Depending on the subpool, the target coverage
305 varied from 70 to 100%, indicating that most of the amino acids were edited. After applying
306 negative selection with the ACC inhibitor haloxyfop they identified multiple seedlings with
307 various alleles at four different amino acids. One previously known amino acid substitution
308 (W2125C) and three novel substitutions (P1927F, S1866F and A1884P) conferred herbicide
309 tolerance (Li et al., 2020).

310 These protein-engineering studies in rice lay the foundations for using CRISPR screens for
311 synthetic biology applications such as protein structure-function studies or for accelerated
312 plant breeding. Using transformation populations of 3,000-15,000 rice calli they were able to
313 obtain evolved allele variants. So far, they have all targeted genes that confer resistance to
314 drugs or herbicides, which greatly facilitates phenotypic selection (Butt et al., 2019; Kuang et
315 al., 2020; Li et al., 2020). Despite these exciting results, significant limitations must still be
316 overcome. Firstly, the mutation spectra of deaminases are largely restricted by the deaminase
317 window and the transitions that can be made. Furthermore, only a restricted number of amino
318 acid substitutions can be generated using base editors (Tong et al., 2019). One main

319 challenge is also to evolve traits that cannot be negatively selected in tissue culture or are
320 quantitative in nature (plant height, drought tolerance, flowering time, etc.). Furthermore, two
321 of the groups were able to use prior knowledge to more specifically target functional domains.
322 While the results of Butt and colleagues clearly demonstrate an increase in efficiency from
323 using a targeted approach, the required knowledge is generally lacking for many genes and
324 species.

325 **DESIGNING A POOLED CRISPR KNOCKOUT SCREEN**

326 Having described the current applications of CRISPR screens in plants, we will now elaborate
327 specifically on the design criteria to carry out a CRISPR knockout screen in plants (Figure 2).
328 We draw from our experiences generating CRISPR libraries as well as those recently
329 published. It is important to first think through the entire workflow before ordering any
330 reagents. What type of editing tool will I use and what is its efficiency? How will I
331 transform/transfect my plants? Can I generate, grow, and analyze a population large enough
332 for a genome-wide screen or is a targeted approach more realistic? If a focused screen is
333 used to investigate specific regulatory pathways or quantitative phenotypes, how will I select
334 the target genes? Are they gene candidates from differential expression analysis,
335 QTL/GWAS, phylogenetic relationships, *etc.*? Once the library is made, how will I phenotype
336 and identify candidate genes and mutations? How will I confirm the 'hits'?

337 All of these questions must have clear answers before starting the screen. Otherwise one
338 might generate a large collection of transgenic materials with no space to grow them, no
339 simple way of knowing whether the phenotype they are seeing is caused by genome editing
340 (or the inverse, why they are not observing any phenotype), nor understanding how
341 mutagenized their population is.

342 **Choice of Editing Tool**

343 A large panel of genome-editing tools is currently available for plants (Zhang et al., 2019b).
344 Changing promoters and Cas9-codon usage are frequently used to increase editing
345 efficiencies (Hanson and Collier, 2018; Zhang et al., 2019b). For most knockout screens,
346 SpCas9 is the simplest choice as most tools have been designed with this enzyme and it is
347 still more active than any of the variants with relaxed PAM requirements (Endo et al., 2019;
348 Legut et al., 2020; Walton et al., 2020; Zhong et al., 2019). The limitation of the NGG PAM
349 does not present considerable issues when targeting most plant genomes for simple

knockouts. However, it may be limiting in some circumstances such as base editing or transcriptional control where there are greater targeting restrictions (specific amino acids or TA-rich regions). Cas9 variants such as xCas9 and Cas9-NG recognize NG, GAA and GAT PAMs and can accommodate a greater variety of targets (Hu et al., 2018; Nishimasu et al., 2018). The target range and indel spectrum is further expanded by Cas12a which recognizes T-rich PAMs and introduces a staggered DNA cut (Tang et al., 2017; Zetsche et al., 2015). Lastly, the DNA repair outcomes may be a factor worth considering. The use of the exonuclease Trex2, in combination with Cas9, has been shown to produce larger deletions than Cas9 alone, and therefore could be used to investigate non-coding RNA sequences where small indels may not result in a significant effect (Čermák et al., 2017; Ho et al., 2014; Tan et al., 2020)

Choice of Screening Format

The method-of-choice for all plant CRISPR screens to date has been stable plant generation with pooled vectors. The main advantages of this approach is that it is relatively simple to implement using existing plant transformation protocols and enables analyzing plant phenotypes over multiple generations, across development and under various environmental conditions. It also allows one to easily isolate causative alleles from the mutagenized population by genetic segregation and/or crossing. Pooled transformations have been so far performed with *Agrobacterium* or direct-DNA delivery via biolistics.

With multiple transformation options available such as electroporation, biolistics, polyethylene glycol- or *Agrobacterium*-mediated transformation, it is important to consider how genome editing reagents are delivered to the cells. Pools of *Agrobacteria* typically result in only one or few bacteria introducing their T-DNAs in any individual plant cell (Depicker et al., 1985). Consequently, if plasmids are pooled, one should expect that ~80% of events will contain a single T-DNA and the rest will contain two, three, or even more different T-DNAs. Such distributions of T-DNAs have been observed in pooled screens (Bai et al., 2019; Jacobs et al., 2017; Kuang et al., 2020). The one report using biolistically delivered DNA suggests that co-transformation is possibly higher using biolistics as compared to *Agrobacterium* (Kuang et al., 2020). Such co-transformations are in many cases beneficial to the researcher as it increases the throughput of the screen and thereby decreases the necessary population size. However, multiple vectors can also make the downstream data analysis more complex.

382 Another important consideration of plant transformation is that transient expression of
383 CRISPR reagents is sufficient to induce inheritable mutations (Jacobs et al., 2017; Kuang et
384 al., 2020; Woo et al., 2015). In the transporter library reported in Jacobs et al., all target loci
385 were checked for indels in all transgenic lines in addition to sequencing the gRNAs. This
386 analysis revealed that 20% of the mutations were induced by gRNAs that were not integrated,
387 suggesting that transient expression of T-DNAs without integration led to these mutations.
388 Likewise, Kuang et al. observed base edits where the corresponding gRNAs were not
389 integrated, leading them to suggest the activity of transiently-expressed T-DNAs. These are
390 important observations as it shows that relying solely on sequencing the gRNAs from pooled
391 transformations will likely miss mutations. The other pooled CRISPR libraries generated in
392 plants (Bai et al., 2019; Liu et al., 2020; Lu et al., 2017; Meng et al., 2017) are likely more
393 mutagenized than reported. It is important to note that this effect might be genotype and/or
394 transformation method dependent. PacBio sequencing all 54 genes in six lines of the LRR-XII
395 library revealed no obvious indels from transiently-expressed T-DNAs (Jacobs et al., 2017).
396 On one hand, the presence of transiently-expressed T-DNAs inducing mutations is beneficial
397 as it can increase the mutagenesis rate within the population. Nevertheless, it means that a
398 significant amount of genotyping and/or crossing will need to be performed on lines of interest
399 to properly associate phenotypes with the underlying genotypes. Alternatively, researchers
400 may wish to identify conditions that limit the transient expression of T-DNAs.

401 Producing large numbers of transgenic plants is also a crucial when performing an *in planta*
402 CRISPR screen. The majority of reports in plants to date have been performed in species that
403 are highly amenable to transformation such as rice or tomato. In maize, Liu et al. (Liu et al.,
404 2020) have reported a *tour de force* by generating over 4,000 independent transgenic maize
405 events. Very few groups will have the capability to generate this screening population in
406 maize or other species recalcitrant to transformation and tissue culture. Fortunately, recent
407 reports have demonstrated that overexpression of morphogenetic regulators such as
408 WUSCHEL2 or BABY-BOOM can greatly improve the throughput to generate transgenic
409 plants in several monocotyledon species by stimulating callus regeneration or by inducing
410 somatic embryogenesis (Lowe et al., 2018, 2016). Thus, the improvement of plant
411 regeneration capabilities might allow scaling up the CRISPR screen.

412 Protoplasts are isolated from multiple plant tissues -such as roots or leaves- after digestion of
413 their surrounding cell walls and easily provide a large source of cells that can be used for

transient assays (Yoo et al., 2007). Genome editing reagents are frequently tested in protoplast systems and the analysis is typically limited to DNA or RNA profiles (Bargmann et al., 2013; Shan et al., 2014). Their ease of isolation from a wide range of plant species and the high numbers of transfection events make them an extremely attractive model, especially for genome-wide screens. Yet there are currently no reported pooled CRISPR screens in plant protoplast systems. One potential issue when using protoplasts could be that as micrograms of plasmids are typically used in protoplast transfections, every single gRNA in the library could enter each and every cell during a pooled transformation. Such a possibility needs to be experimentally determined, but this would defeat the purpose of a screen where the objective is to create a variable population. Thus, protoplasts may be better suited for arrayed-based screens on an automated robotized platform that can transfect large numbers of independent samples (Vanden Bossche et al., 2013).

To overcome these limitations, plant cell suspension cultures might offer an alternative that shows comparable features to lentiviral delivery in mammalian cell cultures: they are easily maintained in aseptic conditions and can display fast growth (18-24 hours doubling time for tobacco BY-2 cells (Santos et al., 2016). Furthermore, cells suspensions can be stably transformed with *Agrobacterium* (Forreiter et al., 1997; Santos et al., 2016), which, as previously mentioned, leads to a majority of cells containing a single plasmids. These features can allow for pooled vector libraries to be transformed, propagated and potentially selected. Although the process of creating a plant cell suspension culture *de novo* might be time consuming (Mustafa et al., 2011), plant cell cultures are currently available for several species (Moscatiello et al., 2013; Santos et al., 2016). Obtaining and using established material might therefore be an efficient strategy for pooled CRISPR screen.

Number of Targets

Perhaps the most critical design criterium is determining the number of genes that can be targeted. Surely everyone would perform a genome-wide screen for their trait of interest if they could, but this is often not practical in whole plants for a number of reasons, primarily transformation efficiency, growth space and reagent cost. Here we present a simplified formula to help researchers approximate the number of genes they can reasonably fit in their pooled CRISPR screen. We will elaborate on each of the variables.

$$\# \text{ of targets} = \frac{\text{Population size} \times \text{Mutagenic efficiency}}{\text{Representation factor} \times \text{Distribution factor}}$$

445 **Population Size**

446 The first variable to consider is the size of a reasonable population one can generate,
 447 maintain and phenotype. Even for a small plant like *Arabidopsis*, generating and phenotyping
 448 thousands of lines requires a significant investment of time and resources. For larger plant
 449 species, such population sizes might become impractical and too expensive very quickly. It is
 450 worth keeping in mind that it is often possible to generate many more transgenic lines than
 451 one can fit in the greenhouse.

452 **Mutagenic Efficiency**

453 This is the percentage of individuals within a population that will carry a knockout mutation.
 454 Several considerations feed into this; the efficiency of the transgene expression, efficiency of
 455 the gRNA, the type of indel generated, the effect of that indel on gene function, and the
 456 inheritability of mutant alleles (if going to the next generation). Researchers should know the
 457 efficiency of their system. Most screens thus far have a mutagenesis frequency ranging from
 458 60 to 80% (Bai et al., 2019; Jacobs et al., 2017; Liu et al., 2020; Lu et al., 2017; Meng et al.,
 459 2017). Having a highly-efficient system is likely to be more important when targeting multiple
 460 genes at once (Bai et al., 2019). Transgenic lines stably expressing Cas9 may increase
 461 efficiency by reducing the typical variation in expression between transgenic events (Osakabe
 462 et al., 2016). In cases where a novel gene editing system is used, the efficiency of the editing
 463 system together with a subsample of gRNAs should be tested and validated on a few target
 464 sites (Li et al., 2020; Liu et al., 2020).

465 **Representation Factor**

466 This considers the number of independent lines that should have mutations in the same
 467 gene(s) to give confidence when correlating genotypes and phenotypes. If a spectacular
 468 phenotype is observed in only one individual line, the researcher should be wary of jumping to
 469 any hypotheses (or calling the patent attorney) regarding the gene(s) mutated in that line. In
 470 Jacobs et al, developmental phenotypes segregated in T₁ lines, but were not associated with
 471 the mutant alleles and thus were likely due to somaclonal effects (Jacobs et al., 2017). Having
 472 multiple lines carrying the same mutated gene(s) all showing the same phenotype is
 473 important for knowing which lines to follow up on. As with most experimental setups, three

independent events is a reasonable number to aim for. However, pooled screens in animal cells typically aim for 300 – 1,000 cells per gRNA (Miles et al., 2016). Similar numbers will likely be needed if such pooled screens are to be attempted in plant cells.

Sampling Distributions

Libraries that are evenly distributed do not contain perfectly equal portions of individual gRNAs. Instead the number of any individual gRNA will fall somewhere on a distribution curve and could vary with more than 10-fold in abundance (Liu et al., 2020; Lu et al., 2017). Sampling distributions are critical when determining the correct population size. As a rule-of-thumb in such binomial sampling distributions, to have 95% confidence of observing a single gRNA at least once, one needs to sample three times the number of gRNAs in the population. Given these parameters, if, for example, one is able to generate a population of 1,000 transgenic events, they can in principle target: $(1000 \times 0.8) / (3 \times 3) = \sim 90$ genes for a single-gene knockout screen.

Selecting Target Sites

Some of the more uncertain aspects of designing a CRISPR screen is selecting the gene targets as well as the target sites on these genes. If a genome-wide screen is not being performed, it is necessary to make a sub-selection of genes. Even with a genome-wide screen, researchers still subpool the gRNAs to make more focused collections (Lu et al., 2017). Datasets such as QTL (Liu et al., 2020), GWAS, or (differentially) expressed genes (Jacobs et al., 2017; Meng et al., 2017) can guide the selection of target genes whereas knowing critical functional domains of target proteins can focus the screen on specific regions (Butt et al., 2019; Kuang et al., 2020; Li et al., 2020). For knockout screens we follow the general recommendations to target constitutive exons (Doench, 2018); avoid regions close to the start site to avoid alternative start codons, or regions close to the stop codon to prevent the creation of truncated proteins, and, if possible, in regions of the gene important for its function. These general guidelines often leave us targeting the middle ~75% of gene coding sequences.

When selecting the target site(s), it is critical to establish the genotyping assays for later steps. Primers to amplify the target regions and/or gRNAs should be tested with the desired sequencing technology before ordering any cloning reagents. While many researchers may

wish to skip this step as testing primers is rather mundane, performing these tests saves more time and limits unnecessary stress and anxiety in later steps. Genotyping errors of a target site due to adjacent homopolymers or amplicons that do not match the reference genome sequence can significantly stall a project. Target sites, or even whole genes, that are not compatible with later genotyping steps should be removed from the library; it makes little sense to target a gene/region if the analysis will be blind to it in later steps.

Guide RNA Library Construction

In plants, only a few genome-scale libraries have been designed and thus, although premade knockout libraries are available for rice (Lu et al., 2017; Meng et al., 2017), building a new library is necessary for other plant species or if a more targeted screen is desired. To increase the chances of generating loss-of-function mutations, target regions on constitutive exons are selected and all possible gRNAs identified. Targets containing homopolymers and/or extreme GC contents (<25% and >80%) are generally avoided because they show lower efficiency on average (Doench et al., 2014; Lu et al., 2017; Shalem et al., 2014; Wang et al., 2014). As researchers are often still left with a large number of potential gRNAs, predicted gRNA efficiency is frequently used to further select those with potential maximum on-target activity. This step can be automated using computational tools such as CRISPR library designer (CLD) which offers the option to use plant genomes as input (Heigwer et al., 2016). However, it is worth pointing out that all gRNA activity tools reported thus far are based on animal cell models and their results in plants have been mixed (Naim et al., 2020). A simple approach to overcome inevitable gRNA inefficiencies is to design three to four gRNAs per target gene (Lu et al., 2017; Shalem et al., 2014). Note that gRNA selection is necessarily linked to the selection of target sites and these two decisions often have to be made in tandem.

Cloning can begin after target and gRNA selection. To start, gRNAs are synthesized as DNA oligo pools. There are currently two types of formats available for oligo pools. Those from array-based synthesis are the least expensive per gRNA and can generate tens of thousands of unique sequences. However there are some drawbacks: The minimum numbers of sequences that must be ordered are usually in the thousands, they require PCR amplification to generate sufficient starting quantities for cloning (care must be taken to avoid amplification bias) and they also have relatively high error rates of 1 in 200 to 1 in 2,000. These considerations make arrayed-based oligos more suitable to large-scale genome-wide screens requiring tens of thousands of unique gRNAs. Standard column-synthesized, de-salted oligos

536 can also be used. Any standard oligo provider can generate very large quantities that are
537 sufficient for cloning and contain few errors (typically < 1 in 2,000). However, they become
538 very expensive when ordering large numbers and often require manual steps for pooling,
539 making this option better suited for smaller-scale screens needing tens to hundreds of
540 gRNAs. However, there is often a dilemma when creating libraries somewhere in the middle
541 of these two extremes. Fortunately oligo providers are now starting to bridge the gap and are
542 offering large numbers of relatively inexpensive pooled, column-synthesized oligos.

543 The oligos or PCR products are then cloned into expression vectors by various methods such
544 as restriction-ligation (Lu et al., 2017), Gibson assembly (Jacobs et al., 2017; Meng et al.,
545 2017) or by homologous recombination (Liu et al., 2020). This can be done either individually
546 or pooled. It is important that the cloning strategy be highly accurate ($\geq 95\%$ correct clones)
547 and efficient. Otherwise large numbers of incorrect clones will reduce the mutagenic efficiency
548 of the library. Large numbers of independent clones are also needed to maintain gRNA
549 distribution. Guide RNA coverage of 25X (Jacobs et al., 2017) or 166X (Shalem et al., 2014)
550 have been used for different libraries, requiring tens of thousands to millions of independent
551 clones. These are very large numbers of clones to generate and this is a step where many
552 researchers can get stuck. Any new cloning strategy should be thoroughly tested and cloning
553 sites with the *ccdB* negative selection marker are highly encouraged. After cloning, the quality
554 of the library is evaluated by Sanger sequencing for single clones or amplicon sequencing for
555 pooled clones. In addition to checking for correct gRNA sequences, the distribution of the
556 library is assessed by analyzing the relative abundance of each gRNA with a histogram,
557 which can be generated with computational tools such as the one developed by Joung et al
558 (Joung et al., 2017). Guide RNAs should be normally distributed without individual gRNAs
559 being overly enriched in the library (Doench, 2018). Those that are enriched for in the *E. coli*
560 library will likely be enriched for in the transgenic population (Liu et al., 2020) and can have an
561 impact on the quality of the screen, if an evenly distributed mutant collection is the goal.
562 Limiting bacterial growth time after gRNA library transformation is also important to limit bias
563 (Joung et al., 2017). When using *Agrobacterium*-mediated transformation, the final pool of
564 bacteria used for plant transformation can also be analyzed (Liu et al., 2020; Lu et al., 2017;
565 Meng et al., 2017). After all quality control measures have been met, the library is ready to be
566 transformed into plant tissues.

567 **Data Acquisition and Screening Validation**

568 The main aim of generating a knockout population is to uncover gene function. At least two
569 approaches can be used to screen a mutant library: 1. Screen for phenotypes and then
570 genotype interesting lines. 2. Genotype everything first, organize the lines into a collection
571 and then phenotype and make associations. Genotyping can be performed by sequencing the
572 integrated gRNAs and/or target genes. A single primer pair is used to PCR amplify the
573 gRNA(s) before submitting the product to sequencing. The identity of sequenced gRNAs are
574 then used as a proxy for the mutated genes as the presence of gRNA does not necessarily
575 imply that the corresponding mutation has been introduced in the generated line (Jacobs et
576 al., 2017; Lu et al., 2017). As noted earlier, transient expression of gRNAs can also lead to
577 target mutations without insertion of the gRNA (Jacobs et al., 2017; Kuang et al., 2020) and
578 thus additional genes may be mutated.

579 To confirm the mutation and assess indel efficiencies, genomic regions surrounding the target
580 site are amplified and sequenced with Sanger or massively parallel sequencing (Fauser et al.,
581 2014). Sequence alignment software such as TIDE, ICE, crispRVariant, CRISPResso2 or
582 amplican can then be used to measure indel efficiencies and determine the mutant alleles
583 (Brinkman et al., 2014; Clement et al., 2019; Hsiau et al., 2019; Labun et al., 2019; Lindsay et
584 al., 2016). It is essential that these genotyping assays are established during the design
585 phase (see above).

586 Following successful phenotyping and genotyping, the association between genetic
587 perturbations and the mutant phenotype needs to be validated. We recommend at least two
588 independent mutant lines using independent gRNAs to validate any observed phenotypes.
589 Alternatively, progeny from at least two independent lines segregating for the phenotype can
590 help confirm causative alleles (Jacobs et al., 2017).

591 The phenotyping of whole organisms in plant CRISPR screens largely complexifies the
592 endeavor compared to animal cell culture systems. At the same time, this presents a
593 significant advantage for the phenotypes that can be uncovered as multiple traits in the
594 mutant collection can be analyzed under a range of environmental conditions (Yang et al.,
595 2020). Accordingly, except for a few CRISPR mutant collections in *Drosophila* (Meltzer et al.,
596 2019; Port et al., 2020), whole organisms have generally not been used in CRISPR screens.
597 Depending on the trait analyzed and the species used, plant phenotyping can be labor

intensive and subjected to important environmental variation when conducted in the field. Using genetic reporter lines as background material for screening can facilitate phenotyping and give further insight on the regulation of particular molecular pathways (Feldman et al., 2019; Marquès-Bueno et al., 2016; Pusapati et al., 2018; Wright and Nemhauser, 2019). Hence it is important to consider how the phenotyping will be performed once the library is generated several months to years later. It may be desirable to perform pilot experiments testing a variety of reporter lines to find the most suitable one.

FUTURE PROSPECTS FOR CRISPR SCREENS IN PLANTS

Since the first reports of a CRISPR screen in mammalian cells in 2014 (Shalem et al., 2014; Wang et al., 2014), the use of CRISPR screens is slowly being adopted by the plant research community. As there are still relatively few study cases, many open questions still remain on the best way to use and implement the technology in a research program. We and others are continuing to develop this technology and we anticipate that in the near future performing a CRISPR screen or ordering a CRISPR library will become as common as performing an EMS screen or requesting T-DNA/transposon insertion lines. In this section we highlight some of the open questions that should be overcome to make the technology easier to use as well as some exciting possibilities that are not currently achievable with existing technologies.

Development of Prediction Tools for gRNA Efficiency and Editing Outcome

Guide RNA design is a key step in setting up a CRISPR experiment and greatly influences the quality of a CRISPR library. Having more active gRNAs improves the efficiency of the system and reduces the size of the necessary population. The results of Bai et al. suggest that a gRNA pre-screening step can increase the mutagenesis frequency of their library in soybean (Bai et al., 2019). However, such a pre-screen becomes less practical as the number of gRNAs increases. Therefore, an *in silico* screening pipeline to select highly-active gRNAs is desirable. Predictive tools have been established in mammalian cell systems and use scoring algorithms to predict on- and off-target activities (Doench et al., 2016, 2014; Hsu et al., 2013). The core rules of gRNA efficiency depends on the position, number and identity of mismatches to the genomic target. While the various models can facilitate gRNA design in mammalian experimental systems, their predictive accuracy in plants is still unclear. In an effort to compare gRNA efficiency scores generated by different algorithms, Haeussler et al. observed substantial variation in the predictive tool performances, suggesting that individual

models are optimized for the dataset and the model species they were generated from and that they are not fully transferable to other experimental systems (Haeussler et al., 2016). Along the same line in plants, Naim et al. compared eight design tools to score gRNAs with experimental editing efficiency and observed little uniformity and predictive accuracy (Naim et al., 2020). This suggests that these models need to be adapted to increase their predictive power for plants. Screening approaches similar to mammalian cell systems (Kim et al., 2018, 2020; Wang et al., 2019) could be conducted to improve the predictability of gRNA design algorithms for plants (Figure 3).

Forecasting the type of mutation generated at a target locus is another desirable component of gRNA design. Genome editing outcomes from CRISPR systems are non-random and are dependent on sequence features adjacent to the target site (Shou et al., 2018; van Overbeek et al., 2016). For example, in most knockout screens it would be desirable to remove the gRNAs that primarily lead to in-frame deletions. In contrast, studies such as Butt et al. could benefit by utilizing gRNAs that only generate in-frame deletions. By analyzing editing outcomes for up to 40,000 gRNA-target pairs from synthetic or endogenous target regions, multiple studies have investigated target sequence determinants and have used these large editing datasets to train deep learning programs (InDelphi, FOREcast or SPROUT) to predict mutational outcomes associated with gRNA sequences (Allen et al., 2019; Leenay et al., 2019; Shen et al., 2018). A large proportion (72%) of editing outcomes obtained from Cas9 activity in a maize CRISPR screen were predicted by the ForeCast model (Allen et al., 2019; Liu et al., 2020). This suggests that although the DNA repair machinery is not identical between mammalian and plant systems, the rules determining editing outcomes are largely transferable (Liu et al., 2020). Together with the development of prediction tools for gRNA on- and off-target efficiency, these tools would provide a valuable resource for designing gRNAs and selecting those that generate the desired type of mutation.

Recording Gene Editing Outcome at the Single-Cell Level

The emergence of single-cell sequencing technologies has revolutionized the resolution at which cell identities can be characterized (Macosko et al., 2015; Tanay and Regev, 2017). By analyzing and integrating regulatory interactions of individual cells from a tissue, gene regulatory networks underlying dynamic developmental processes such as cell differentiation can be inferred (Efroni et al., 2015).

660 Microfluidic systems have enabled processing large numbers of single cells and have
661 successfully been applied to plants by multiple independent groups to profile root tissues
662 (Denyer et al., 2019; Jean-Baptiste et al., 2019; Shulze et al., 2019; Zhang et al., 2019).
663 Profiling *Arabidopsis* single root cells revealed new cell type marker genes as well as how cell
664 differentiation trajectories proceed from the proximal meristem to the differentiation zone
665 (Denyer et al., 2019; Shulze et al., 2019). The influence of environmental factors such as
666 sucrose or heat shock on cell identity has also been recorded (Jean-Baptiste et al., 2019;
667 Shulze et al., 2019). Together these studies firmly established single-cell profiling protocols
668 and analysis pipelines for *Arabidopsis* roots and now enable plant functional genetics at high
669 resolution.

670 Combining gene perturbations with single-cell profiling as a phenotypic readout will allow
671 probing gene function in precise cellular contexts (Tanay and Regev, 2017). Multiple reports in
672 mammalian cells have established experimental pipelines as well as computational
673 frameworks for conducting CRISPR screens followed by single-cell sequencing (Perturb-seq,
674 CROP-seq, CRISP-seq) (Adamson et al., 2016; Datlinger et al., 2017; Dixit et al., 2016). In
675 these studies, the gRNA library is cloned on a vector that carries barcodes to label individual
676 gRNAs which can subsequently be detected by RNA sequencing. By targeting a set of
677 regulatory factors and recording the resulting transcriptional changes at the cellular level, this
678 method has allowed the investigation of the regulatory signature of transcription factors as
679 well as cell cycle genes on cellular programs in various cell systems (Adamson et al., 2016;
680 Dixit et al., 2016).

681 As both CRISPR screens and single-cell sequencing have been established in plants, we
682 envision that these methods will be combined to further characterize genetic programs in
683 plants (Figure 4). However, several challenges remain for the application of this technology to
684 plants. The heterogeneity in plant cell size and the requirement to digest cell walls strongly
685 decreases the efficiency of the single cell isolation procedure. To this day, although the
686 number of cells analyzed by single-cell sequencing in *Arabidopsis* roots varies from 3,000 to
687 12,000 (Denyer et al., 2019; Jean-Baptiste et al., 2019; Shulze et al., 2019; Zhang et al.,
688 2019a), Perturb-seq experiments analyze 26,000 to 104,000 cells (Dixit et al., 2016). Thus,
689 scaling up the number of cells analyzed will be crucial for applying these methods in plants.
690 This could be accomplished by using cell suspension cultures or protoplasts (Moscatiello et
691 al., 2013; Yoo et al., 2007) as scaling up their culture condition would generate enough

692 material to transform Cas9 and a gRNA library that would be processed for single-cell
693 sequencing (Figure 4). Finally, the use of a computational framework such as the one
694 developed in Dixit et al. might also allow downscaling the number of cells analyzed to identify
695 core regulatory signatures resulting from genetic perturbations (Dixit et al., 2016).

696 **Combinatorial CRISPR Screens**

697 The multiplexing capabilities of the CRISPR-Cas system combined with its simple
698 programmability allows researchers to simultaneously target multiple genes to investigate
699 genetic interactions or redundant genes. In mammalian and yeast cell cultures, combinatorial
700 CRISPR screens have been conducted to test genetic interactions in a high-throughput
701 manner, systematically interrogating thousands of pairwise genetic interactions using paired-
702 gRNA libraries and facilitating the dissection of complex genetic networks (Gonatopoulos-
703 Pournatzis et al., 2020; Guo et al., 2019; Najm et al., 2018; Roy et al., 2018; Shen et al.,
704 2017; Wong et al., 2016; Zhao et al., 2018, 2019).

705 In contrast, large-scale combinatorial CRISPR screens have not yet been reported in plants.
706 Although CRISPR-based single gene knockout collections provide a valuable resource for
707 plant functional genetics (Liu et al., 2020; Lu et al., 2017; Meng et al., 2017), they do not
708 provide more insight than EMS mutagenesis or T-DNA/transposon insertion collections for
709 investigating gene function and their putative interaction partners. Furthermore, polyploidy is a
710 common phenomenon in plants that can lead to extensive functional redundancy, making the
711 analysis of genetic screening challenging, especially when multiple multi-allelic genes are
712 targeted and segregate over generations (Comai, 2005; Zaman et al., 2019). Combinatorial
713 CRISPR screens have the potential to overcome these challenges by simultaneously
714 targeting all genes in a particular family, regulatory module, or metabolic pathway of interest.
715 Additionally, by stacking multiple gene targets onto a single plasmid, far fewer individuals
716 need to be generated to obtain mutations in all target genes.

717 Thus far, two reports have laid the foundation for combinatorial CRISPR screens in plants
718 (Bai et al., 2019; Jacobs et al., 2017). In principle, several strategies can be used to
719 simultaneously target genes (McCarty et al., 2020). First, individual genes can be targeted by
720 co-transformation of two or more plasmids carrying different individual gRNAs (Bai et al.,
721 2019). This results in transgenic lines containing primarily one gRNA, however two, three, or
722 even more gRNAs can integrate and lead to mutations. As the integration of plasmids is a

723 random process, populations of plants containing various combinations of indels can be
724 produced.

725 In a second approach, multiple guides can be expressed from a single plasmid (Jacobs et al.,
726 2017; Ma et al., 2015; Zhang et al., 2016). This can either be done by expressing each gRNA
727 with an individual promoter or as a single RNA transcript. Polycistronic RNA transcripts can be
728 processed by components of the native CRISPR-Cas system such as Cas12 (Hu et al., 2019;
729 Pu et al., 2019; Wang et al., 2017) or they can be processed by flanking the gRNAs with an
730 RNA cleavage site, e.g. self-cleaving ribozymes (He et al., 2017; Tang et al., 2016), Csy4
731 recognition sites (Čermák et al., 2017; Yingzhu Liu et al., 2019), or tRNA processing signals
732 (Čermák et al., 2017; Xie et al., 2015) (Figure 4B). This method requires the creation of large
733 gRNA libraries as all gRNA combinations need to be cloned and can be facilitated using
734 multiplexed cloning strategies (Čermák et al., 2017; Jacobs et al., 2017). The position of the
735 gRNA in a guide array may have an impact on its efficiency when expressed from individual
736 promoters (Jacobs et al., 2017). A positional-dependent effect was also observed using a
737 polycistronic transcript containing ten gRNAs and processed by Csy4 (Kurata et al., 2018).
738 Both reports observed that the first guides generated mutations at higher efficiencies than the
739 last, suggesting that the position of the gRNA on the array impacts its efficiency (Kurata et al.,
740 2018). However, other reports have not observed position effects (Čermák et al., 2017;
741 Lowder et al., 2015) suggesting that gRNA position effects might be dependent on the vector,
742 species or the transformation method used.

743 Lastly, designing nonspecific gRNAs that target closely related sequences can increase the
744 number of targeted genes without delivering additional gRNAs (Bai et al., 2019). The
745 bioinformatics tool CRISPyS can be used to design gRNAs that target sets of genes
746 (preferably from the same gene family) based on the observation that specific mismatches
747 between the gRNA and target are tolerated (Hyams et al., 2018). This strategy is particularly
748 suited for investigating genetic redundancy within gene families or gene function in polyploid
749 plants (Bai et al., 2019) but can be harder to implement in species with less sequence
750 homology, such as *Arabidopsis*.

751 Although the genetic tools to generate multiplexed gRNA libraries are available, many
752 challenges remain to conduct high-throughput pooled, combinatorial CRISPR screens in
753 plants. First, the ideal size of the screen (such as the number of target genes per pool/batch

754 and number of gRNAs per vector) needs to be empirically determined. While using smaller
755 gene pool sizes and fewer gRNAs per vector might facilitate downstream analysis, this will
756 also reduce the throughput of the screen. Another possibility is that the chance for lethality or
757 severe phenotypes might increase with higher-order knockouts. Approximately 10% of all
758 Arabidopsis genes are essential (Lloyd and Meinke, 2012), and as ever more gRNAs are
759 stacked on a plasmid, chances become higher of obtaining highly mutagenized lines (or not, if
760 very severe) that may be too strongly affected to be useful in a screen. Therefore, a balance
761 will likely need to be struck between the efficiency of generating higher-order mutants and
762 those that will provide useful phenotypes in a screen.

763 Furthermore, the combinations of gRNAs (genes) to target is an important consideration when
764 multiplexing. For large libraries, it is not possible to target every gene in every possible
765 combination. Even with the most efficient transformation systems, it is difficult to generate the
766 numbers of independent events required to make all higher-order mutants. For a genome with
767 30,000 protein coding genes, there are $n(n-1)/2$ or 450 million possible pairwise combinations
768 (Figure 4B). Even for a gene family of modest size of 20 family members, there are 190
769 possible pairwise combinations. Thus, the numbers of individuals quickly become impossible
770 for most systems when higher-order combinations are considered.

771 Another critical challenge is the association of genotypes and phenotypes in complex mutant
772 collections resulting from multiplexed gene knockout (Costanzo et al., 2019). This has so far
773 been done by manually interpreting the data as there are currently no established automated
774 analysis methods in plants for higher-order libraries (Bai et al., 2019; Hauser et al., 2013;
775 Jacobs et al., 2017; Zhang et al., 2018). There is a need for an efficient method to link genes
776 and combinations thereof to observed phenotypes and to calculate the contribution of the
777 allele to the phenotype. Ideally, such method could generate a function from the data
778 gathered by the combinatorial CRISPR screen that could predict combinatorial gene effects
779 not identified in the screening population (Figure 4B) (Liu et al., 2019b). High-throughput
780 genotyping platforms have been established and the development of robotized phenotyping
781 platforms coupled to association mapping analysis methods could also be used to correlate
782 complex traits with mutant genotypes (Yang et al., 2020). Although these methods are not
783 directly related to CRISPR methodologies, we anticipate that their progress and integration

784 with genetic screens will greatly advance the potential of CRISPR screens for plant functional
785 genetics.

786

787 **CONCLUSIONS**

788 The implementation of large-scale CRISPR screens is starting to reveal the great potential of
789 this method for plant functional genomics as well as for synthetic biology through targeted, *in*
790 *planta* gene engineering. Thus far, it has been largely applied to generate knockout mutant
791 collections or for diversifying gene sequences (Kuang et al., 2020; Li et al., 2020; Liu et al.,
792 2020; Lu et al., 2017; Meng et al., 2017; Rodríguez-Leal et al., 2017). Together, these studies
793 provide a general framework for designing and conducting CRISPR screens in various plant
794 species and also give insight on the current limitations. One of the main challenges is to
795 transform and maintain whole plant mutant collections over multiple generations, which, in
796 contrast to cell cultures, requires significantly more time and resources. On the other hand,
797 the ability to grow and perturb mutants throughout development is a tremendous advantage
798 for uncovering a wide range of genetic effects and should be fully exploited by the plant
799 research community. Strategies to accelerate the creation of transgenic plants (Maher et al.,
800 2020) or to shorten generation time in crops (Watson et al., 2018) will greatly facilitate the
801 construction and screening of CRISPR libraries.

802 Based on established methods in animal cell cultures, we also envision that CRISPR screens
803 could be combined with machine learning to design gRNAs with higher efficiency in plants
804 and to predict genome editing outcomes, thereby increasing the mutagenesis efficiency and
805 decreasing the necessary population size. Furthermore, in combination with single-cell
806 genomics, CRISPR mutant collections could be used to map gene regulatory networks at high
807 resolution in response to environmental or developmental cues.

808 Finally, the multiplexing capabilities of CRISPR genetic tools opens new avenues to
809 investigate molecular processes that could hardly be approached with classical screening
810 methods. Combinatorial gene knockout can interrogate gene function of functionally
811 redundant gene families or in polyploid plants. Hypothesized gene regulatory networks
812 inferred from genomic datasets could be tested and their models further refined (Krouk et al.,
813 2013). Taken together, we anticipate that the continued development of CRISPR screens will
814 tremendously increase the pace of plant functional genetics and synthetic biology.

816 **Author contributions**

817 C.G., W.D. and T.B.J. conceived and wrote the manuscript.

818

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820

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1384 **Tables**

1385

1386 **Table 1. Comparative analysis for genetic screening in plants**

1387 References: ⁽¹⁾ Henry et al., 2014; ⁽²⁾ Alonso 2003; ⁽³⁾ Schwab 2006; ⁽⁴⁾ Tang et al., 2018

1388 SNV: Single nucleotide variant.

Method	Genetic perturbation	Mutagenesis induction	Off-targets	Size mutant population	Ease of gene isolation	Overcomes redundancy
EMS-TILLING	SNV	untargeted	high (> 100 / line) ⁽¹⁾	small to medium	-	-
T-DNA insertion	insertion	untargeted	low (1.5 insertion / line) ⁽²⁾	large	+	-
amiRNA	transcriptional repression	targeted	low and predictable ⁽³⁾	large	++	+
CRISPR	indels, SNV, transcriptional activation and repression	targeted	low and predictable ⁽⁴⁾	large	++	+

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1391 **Table 2. Characteristics for CRISPR screening in plants**
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Screening purpose	Editing tool	Species	gRNA library size	Number of target genes	Number of gRNAs per vector	Mutant collection size	Reference
Gene knockout	SpCas9	Tomato	165	54	1	31 (T ₀)	Jacobs et al 2017
	SpCas9	Tomato	36	18	3	59 (T ₀)	Jacobs et al 2017
	SpCas9	Rice	88,541	34,234	1	91,004 (T ₀)	Lu et al, 2017
	SpCas9	Rice	25,604	12,802	1	14,000 (T ₀)	Meng et al, 2017
	SpCas9	Soybean	70	102	1	407 (T ₀)	Bai et al, 2019
	SpCas9	Maize	1,368	1,244	1	4,356 (T ₀)	Liu et al, 2020
Sequence diversification	SpCas9	Tomato	8	1 (CLV3)	8	1,152 (F ₁)	Rodriguez-Leal et al, 2017
	SpCas9	Tomato	8	1 (S)	8	326 (F ₁)	Rodriguez-Leal et al, 2017
	SpCas9	Tomato	8	1 (SP)	8	81 (F ₁)	Rodriguez-Leal et al, 2017
	SpCas9	Rice	119	1 (SF3B1)	1	6 (T ₀)	Butt et al, 2019
	ABE and CBE	Rice	63	1 (OsALS1)	1	113 (T ₀)	Kuang et al, 2020
	STEME-1 STEME-NG	Rice	200	1 (OsACC)	1	19 (T ₀)	Li et al, 2020

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Figure 1: CRISPR-Cas genome editing tools

(A) Cas9-gRNA complex targeting DNA complementary to the gRNA sequence 3' of the protospacer adjacent motif (PAM). After imperfect DNA repair, nucleotide deletions or insertions can be generated at the target loci. **(B)** Base editors are built by the fusion of Cas9 (D10A) (**nCas9**) or Cas9 (D10A H840A) (dCas9) to cytidine or adenine deaminase domains. Cytidine base editors (CBE) preferentially introduce C to T mutations while adenine base editors (ABE) catalyze A to G mutations. Base editors deaminate nucleotides in a restricted editing window (green nucleotides). **(C)** CRISPR interference (CRISPRi) or CRISPR activation (CRISPRa). dCas9 is fused to transcriptional repressors or activation domains and can modulate RNA levels. Red triangle: nuclease-cutting site. D10A and H840A are Cas9 mutations inactivating nuclease domains.

Library design and preparation

Identify target regions

Target 1
Target 2
...

Oligo synthesis and amplification

Cloning gRNA library in vector system

Plant transformation and selection (T₀ generation)

Analysis and validation

Phenotyping (T₀-T₃ generation)

WT	1	2
Line	Trait1	Trait2
WT	10	10
1	5	10
2	10	5
...		

Amplicon sequencing

Validation genome editing

	gRNA	PAM
Ref	CGATTTCGATTCCTTGATTCCGG	
% editing	CGATTTCGATTCCTTGATTCCGG	
	CGATTTCGATTCCTTGATTCCGG	
	CGATTTCGATTCCTTGATTCCGG	
	CGATTTCGATTCCTTGATTCCGG	
	CGATTTCGATTCCTTGATTCCGG	

Design parameter

- Number of target genes
- Number of gRNA / gene
- gRNA on-target score
- gRNA off-target prediction
- Efficiency of editing system

- Editing tool used
- Cloning system used

- Stable or transient transformation?
- Size of mutant collection?

- Number and type of traits to analyse
- Use of marker/reporter line?

- Method to associate genotype with phenotype

Quality control

- Validate amplicons for selected targets

- Library coverage
- Library uniformity
- Sequence error rate

- Transformation efficiency
- Coverage library in plant mutant collection

- Library coverage
- Library uniformity

- Editing efficiency
- Confirmation of phenotype for positive controls
- Confirmation of mutant phenotype with independent gRNA and/or lines

Figure 2: Workflow and design parameters for CRISPR knockout screening in plants

A CRISPR knockout screen starts with the design and the preparation of the gRNA library. The size of the library is defined based on the final population size. Genotyping assays must be designed and tested for the eventual genotyping of the mutant population. Once the design is done, the experimental part of the screen starts. The Cas enzymes and gRNA library are cloned into appropriate plasmids and the library is evaluated for gRNA distribution and coverage. The library is then transformed into the target tissues and mutant plants recovered. Plants in the primary or subsequent generations can then be selected based on their phenotype and their DNA is isolated, amplified and sequenced. Alternatively, the entire collection may be genotyped resulting in a population with known mutations and/or gRNAs. The resulting sequences are analyzed using sequence alignment software to associate phenotype with genetic perturbations. Finally, the causal mutation is confirmed using independent genome editing assays and functional characterizations. We provide key design parameters to be considered as well as some quality control steps.

Figure 3: Development of gRNA prediction tools in plants using CRISPR screens

Prediction tools for gRNA efficiency can be generated by probing the activity of gRNA libraries. In the arrayed strategy, protoplasts are isolated, arrayed and transfected with a ribonucleoprotein (RNP) complex (Cas-gRNA) or with expression vectors. DNA is then isolated from individual transfections and analyzed using amplicon sequencing. Alternatively, a pooled strategy offers the advantage to be easily scalable for large datasets. A library of synthetic gRNA-target sites is built on a common vector backbone, pooled and transformed into plants. Mutagenesis of the transformed vector occurs in plant cells and can be recorded using a common primer pair that binds backbone sequences of the vector and can thus be used to amplify and record target regions for the whole library followed by sequencing. For the arrayed as well as for the pooled strategy, sequences of mutated target sites and gRNAs are used to train a machine-learning algorithm to generate gRNA design rules and a scoring system.

Figure 4: Mapping and characterization of plant gene regulatory networks using CRISPR screens

(A) CRISPR screen followed by DROP-seq (Macosko, 2015) can decipher gene regulatory networks at single-cell resolution. Vectors encoding Cas9 and a library of barcoded constructs carrying gRNAs are transformed into plant protoplasts or cell suspension cultures. Protoplasts are then loaded on a microfluidic device and encapsulated with barcoded beads for single-cell sequencing. Single-cell profiles are generated and associated to individual gRNAs, thereby allowing the investigation of transcriptional response upon gene perturbation. These profiles are used to infer gene regulatory networks. **(B)** Multiplexed CRISPR screens can characterize redundant gene function or gene interactions. The number of possible gRNA combinations is first assessed and fitted to the desired size of the mutant population. A multiplexed gRNA library is then built and transformed into plants to generate a mutant collection. Individual lines are then genotyped and phenotyped and generate a datasets that can be used to associate phenotype with causal gene perturbations or to infer gene interaction maps.

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