CRISPR/Cas-mediated genome editing for the improvement of oilseed crop productivity

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Abstract

The demand for vegetable oils is increasing at a rapid pace due to our ever-expanding population, growing global affluence, changes in dietary choices, and the need for renewable plant-derived resources. However, oilseed production is negatively impacted by unpredictable environmental conditions caused by climate change, as well as associated increases in disease and pest infestations. Unfortunately, while conventional breeding techniques have been used to provide gains in terms of oilseed yields, they are often imprecise and lengthy processes. Crops derived from transgenic approaches, on the other hand, have proven difficult to get to market due to negative public perception and onerous regulatory requirements. Genome editing, primarily using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas) platform, is a relatively recent addition to our plant breeding toolkit that allows the rapid generation of precise targeted genetic changes that can be indistinguishable from spontaneous mutations. In addition, the resulting plants can be made transgene-free with relative ease. While genome editing has been successfully used to modify a plethora of genes in the model plant *Arabidopsis thaliana*, the technology is only just taking off in oilseed crop species. This review discusses advances that have been made to date using CRISPR/Cas-mediated genome editing of oilseed crops to improve plant productivity under favorable and sub-optimal environmental conditions, leading to increased seed yields or reduced losses. Furthermore, we also examine potential avenues for future enhancements in these traits using this molecular breeding tool.

I. Introduction

Plant-derived oils are an integral part of the human diet, comprising a large proportion of our daily caloric requirement. In addition, they also serve as raw material for various industrial products such as detergents, plastics, waxes, cosmetics and paints, as well as a supplement for animal feed and a renewable energy resource (Wittkop *et al*., 2009). While palm (*Elaeis guineensis*) provides the largest single source of vegetable oil globally, oilseed crops such as soybean (*Glycine max*), rapeseed/canola (*Brassica napus*), sunflower (*Helianthus annuus*), peanut (*Arachis hypogaea*) and cotton (*Gossypium hirsutum*) together provide more than half of the world supply (United States Department of Agriculture Foreign Agricultural Service, 2020).

The demand for vegetable oils is growing steadily due to our expanding population, increasing global affluence, changes in dietary choices, and the need for more renewable plant-derived resources (Villanueva-Mejia and Alvarez, 2017). Since a large proportion of our current vegetable oil supply derives from oilseed crops, substantial improvements in seed oil yield will be required to fulfil this demand. However, achieving such yield improvements will be complicated by the fact that oilseed production can be hindered by climate change-related environmental effects, as well as associated increases in disease and pest infestations, which are all likely to become more problematic in coming years (Jaradat, 2016; Raman et al., 2019). Therefore, multiple avenues could theoretically be taken to achieve enhanced oilseed yields (either alone or in combination), such as increasing seed oil content, enhancing seed yields, or reducing seed yield losses caused by factors such as pod/silique shattering, weed overgrowth, and abiotic/biotic stress resilience (Diepenbrock, 2000; Valantin-Morison and Meynard, 2008).

Conventional breeding techniques, including artificial selection, hybridization and induced mutagenesis, have been employed previously in the development of oilseed crop cultivars; however, each of these methods are complicated by the polyploid nature of most

oilseed crops and require extensive labour and time investments to achieve improvements (Yang *et al*., 2017). In order to meet demands for seed oil in the coming decades, the use of advanced molecular breeding techniques as complementary breeding tools would therefore be highly beneficial to expedite the pace of crop improvement programs. While transgenic approaches have been applied successfully in many oilseed crops to improve a wide range of traits to date (e.g., Meesapyodsuk *et al*., 2018; Na *et al*., 2018; Shah *et al*., 2018; Kim *et al*., 2019; Wang *et al*., 2019a), only a small number of these plants have made it to market due to poor public perception, as well as the exorbitant cost and duration of existing regulatory processes (Mall *et al*., 2018). Indeed, with the exception of a small number of oilseed cultivars, including those exhibiting high lauric acid (12:0; Laurical^{TM} canola, Monsanto) or oleic acid (18:0; e.g., Vistive® Gold soybean, Monsanto) seed oil, or drought tolerance (HB4® soybean, Verdeca), the vast majority of transgenic oilseed cultivars that have been commercialized thus far have included only two traits (herbicide tolerance and/or insect resistance).

Genome editing using the clustered regularly interspaced short palindromic repeats/CRISPR-associated protein (CRISPR/Cas) platform is a relatively recent addition to our plant breeding toolkit, and is particularly valuable in polyploid species (e.g., Mohanta *et al*., 2017) for its ability to elicit targeted bi-allelic/homozygous mutations in multiple alleles. In its simplest form, CRISPR/Cas requires a Cas nuclease, which elicits a double-stranded DNA break (DSB), as well as a short single guide RNA (sgRNA) that includes an approximately 20 nucleotide user-defined sequence responsible for guiding Cas to a specific, pre-determined chromosomal locus upstream of a Cas-dependent protospacer-adjacent motif (PAM). Various Cas nucleases derived from numerous bacterial species, including Cas9 (e.g., Kaya *et al*., 2016; Li *et al*., 2018a), Cas12a (formerly known as Cpf1; e.g., Zaidi *et al*., 2017) and Cas12b (Ming *et al*., 2020), as well as a number of engineered Cas variants with alterations in their PAM

recognition domains and/or specificities (Raitskin *et al*., 2019), have been used effectively in plants to date, with the most commonly used being Cas9 from *Streptococcus pyogenes* (PAM = 5' – NGG – 3'). DSBs produced by these Cas nucleases are most frequently repaired by the plant's inherent non-homologous end-joining (NHEJ)-based repair mechanism, which leads to a small insertion or deletion (indel) at the target site. This yields a genetic change that can be indistinguishable from those occurring spontaneously or through induced mutagenesis, and typically knocks out or knocks down the function of the edited gene (reviewed by Subedi *et al.*, 2020; Figure 1). Another DNA repair pathway, termed homology-directed repair (HDR), can also be harnessed to achieve targeted transgene insertions and allele replacements in plants (Shi *et al*., 2016); however, this technology has been limited by low efficiencies and difficulties associated with the delivery of the DNA repair template that must accompany the Cas protein and sgRNA in this instance (Chen *et al*., 2019).

CRISPR-based technology is evolving at a very rapid pace, and a number of alternative derivatives to standard NHEJ- and HDR-based platforms have now been developed. For example, the fusion of transcriptional repressor, activator or demethylase domains to deactivated Cas9 can provide transcriptional or epigenetic regulation (Lowder *et al*., 2015; Gallego-Bartolomé *et al*., 2018), and the use of the Cas13 single-stranded RNA nuclease elicits post-transcriptional regulation of gene expression (Wolter and Puchta, 2018). In addition, systems involving the use of Cas enzymes that have been modified to cut only a single strand of a DNA duplex (nickase activity), such as base-editing (Zong *et al*., 2017; Li *et al*., 2018b) and prime-editing (Anzalone *et al*., 2019), where specific nucleotide changes can be made at targeted locations, have also been shown to be functional in plants (Lin *et al*., 2020; Wu *et al*., 2020a; Figure 1) and improvements in the efficiencies of these tools are almost certainly on the horizon.

While all of these techniques have value, one of the most important considerations in their implementation will be minimizing regulatory burden with respect to new cultivars. Several countries, including the United States, currently do not consider transgene-free NHEJ-derived genome edited plants to be 'GMO', thereby eliminating the need for regulatory measures and facilitating commercialization (Scheben and Edwards, 2018). However, the global regulatory landscape is incredibly complex, and many countries are currently in a state of flux in an attempt to modernize their guidelines and encompass crop varieties developed using genome editing into their policy frameworks (reviewed by Metje-Sprink *et al*., 2020; Parrott *et al*., 2020; Schulman *et al*., 2020). Although global synchrony among countries may not be seen in the short term, it is likely that, in at least some countries, the relaxation of regulations for genome edited crops will hinge upon a lack of foreign, transgenic DNA. In certain instances, such as with transcriptional repression and activation, or RNA-mediated editing, the presence of a transgene expressing Cas and the sgRNA is a requirement. However, in the case of basic NHEJ-mediated editing, as well as base- and prime-editing, plants can be made transgene-free once the targeted edit has been achieved, either by segregating out the transgene or by removing the transgene through programmed death of pollen/embryos containing the transgene (He *et al*., 2018). Alternatively, transgene-free genome edited plants can be made through the direct introduction of Cas/gRNA ribonucleoprotein (RNP) complexes, which degrade rapidly in plant cells (Liang *et al*., 2017; Andersson *et al*., 2018; Park *et al*., 2019). As such, these techniques in particular could hold a great deal of promise in terms of the future improvement of crops.

Although a plethora of CRISPR/Cas-related research has been carried out in the model species *Arabidopsis thaliana* to date (e.g., Gao *et al*., 2016; Jiang *et al*., 2014; Li *et al*., 2014; Miki *et al*., 2018; Xu *et al*., 2018), attempts to edit oilseed crop species such as soybean (Cai *et al*., 2015; Li *et al*., 2015), *Brassica spp*. (Yang *et al*., 2017), cotton (Zhang *et al.*, 2018c) and

146 camelina (Jiang *et al*., 2017) have lagged until recently. In this review, we summarize current
147 progress in the utilization of CRISPR/Cas-mediated genome editing achieved through NHEJ or
148 base-editing to elicit improvements in plant productivity in the form of enhanced seed yields and
149 reduced yield losses. Such improvements can be achieved through alterations in plant
150 architecture and seed characteristics, as well as superior resistance to seed shattering and
151 diseases, herbicide tolerance, and climate change resilience (for a recent review of genome
152 editing for enhanced oilseed lipid content and composition see Subedi *et al*., 2020; Figure 2). We
153 also examine additional gene targets that may have potential to be utilized for the improvement
154 of these traits using genome editing methods that could lead to transgene-free plants. Since the
155 vast majority of genome editing attempts thus far have involved NHEJ-mediated mutations,
156 which typically result in the functional knock-down or knock-out of the targeted gene, we have
157 mainly focused on potential targets that have been shown previously to act as negative regulators
158 within related pathways. However, we also consider the prospect of more recent CRISPR-based
159 technologies, such as prime-editing, as a means of contributing to our ability to meet demand for
160 seed oil in the future.
161
162 **II. Increasing seed number and size**
163 Seed size and number are major determinants of the total yield for oilseed crops per unit area.
164 Both are complex traits, and while genetic/genomic information regarding their regulation is
165 now beginning to accumulate, the precise molecular mechanisms governing them remains
166 unclear (Li *et al*., 2019a). However, progress is being made in this area and several negative
167 regulators of various morphological and physiological parameters, including silique
168 characteristics, stem/inflorescence branching, and cell proliferation within developing seed
169 tissues, have been found to be beneficial in this context (Figure 2).

A. Modification of plant architecture

The manipulation of plant architecture or growth characteristics can significantly enhance crop adaptability and seed yield (Teichmann and Muhr, 2015), typically through increased seed number. A particularly interesting group of genes that appears to function in this capacity includes *CLAVATA* homologs (*CLV1*, *CLV2* and *CLV3*), which play a prominent role in the determination of plant body form by directing meristematic stem cells towards organ initiation (Schoof *et al*., 2000; Yu *et al*., 2003). In line with this, mutations affecting the function of any of the three *CLV* genes typically leads to a delay in organ formation, along with a resulting overgrowth of meristematic cells and the production of extra floral organ whorls (Clark *et al*., 1993; Clark *et al.*, 1995; Kayes and Clark, 1998; Schoof *et al*., 2000). In terms of agronomic performance, this effect can provide desirable outcomes such as increased flower number and alterations in fruit morphology. For instance, it has been suggested that a partial loss-of-function mutation of tomato *CLV3* played a role in the increase in fruit size and locule number that has occurred during domestication (Xu *et al*., 2015). Similarly, increased silique locule number, which translates into higher seed yields, seen in certain genotypes of *Brassica juncea* and *Brassica rapa* has been attributed to mutations in *CLV1* or *CLV3* homologs, respectively (Yadava *et al*., 2014; Xu *et al*., 2017a).

However, multilocular mutants derived from spontaneous or induced mutagenesis have not been identified in *B. napus*, which is likely a direct result of its allotetraploid nature and the associated minute probability of achieving simultaneous random mutations across multiple gene copies (Yang *et al*., 2018). To overcome this challenge, NHEJ-mediated mutations in both subgenome copies of *CLV1*, *CLV2* and *CLV3*, respectively, were realized in *B. napus* using CRISPR/Cas9 (Yang *et al*., 2018), which is known for its ability to yield a range of

194 mutation dosages across multiple gene copies in polyploid species (e.g., Wang *et al*., 2014a;
195 Braatz *et al*., 2017). While plants with bi-allelic/homozygous mutations in *BnCLV1*, *BnCLV2*
196 and *BnCLV3* gene copies, respectively, were found to exhibit a multilocular silique phenotype,
197 this effect was more variable and less stable in lines bearing mutations in *BnCLV1* and
198 *BnCLV2* than in those with mutations in *BnCLV3*. Lines bearing mutations in *BnCLV3* also
199 displayed a significant increase in the number of seeds per silique, seed weight and the number
200 of leaves per plant than wild-type controls (Yang *et al*., 2018), which suggests that the genome
201 editing-mediated modulation of *CLV3* homologs could be a promising strategy for the future
202 improvement of oilseed yields.
203 Increasing stem branching, and hence silique production, can also prove beneficial with
204 respect to boosting seed yields. The *MORE AXILLARY GROWTH 1* (*MAX1*) gene, which
205 encodes a cytochrome P450 monooxygease (CYP711A1) that is involved in strigolactone
206 biosynthesis and acts as a repressor of vegetative axillary bud outgrowth in a wide range of
207 plant species (e.g., Lazar and Goodman, 2006; Zhang *et al*., 2018a; Zheng *et al*., 2020), may
208 provide an ideal target in this context. Indeed, the simultaneous CRISPR/Cas9-induced NHEJ-
209 based knock-out of all four *B. napus BnaMAX1* alleles has been found to result in a semi-dwarf
210 phenotype with significant enhancements in both branch and silique number, as well as seed
211 yields (Zheng *et al*., 2020). Above and beyond seed yield gains, the short stature of these
212 plants could also be beneficial in terms of reducing the risk of lodging and facilitating
213 harvesting (Zheng *et al*., 2020). Since the function of *MAX1* homologs appears to be well-
214 conserved among plant species, especially in dicotyledonous plants, it is possible that this
215 approach could yield similar results in a range of oilseed species. However, strigolactones are
216 also known to function as host recognition signals for symbionts in the rhizosphere, and the
217 knock-down of *GmMAX1a* in soybean has been found to lead to decreased nodule numbers (ur

Rehman *et al*., 2018), which implies that this strategy may not be ideal for leguminous oilseed species.

The mutation of *APETALA 1* (*AP1*), which encodes a well-known floral homeotic gene that plays an important role in floral meristem establishment and the determination of floral organ identity (Irish and Sussex, 1990), has also been found to increase seed number in Brassicaceae species through effects on plant architecture and flowering (Shah *et al*., 2018). Indeed, the ethyl methylsulfonate (EMS)-induced mutation of a single *B. napus Bna.AP1.A02* gene (of six paralogs total) led to significant increases in plant height, branch length, and branch number compared to wild-type plants. Increased seed yields in these mutant plants were also observed, resulting from a significant enhancement in the number of siliques and seeds per plant (Shah *et al*., 2018) that were likely due to the production of ectopic floral buds and enhanced shoot branching. These effects are characteristic of *ap1* mutants in Brassicaceae species (Bowman *et al*., 1993), and while concomitant increases in seed weight cannot be ruled out, this was not assessed in this study (Shah *et al*., 2018).

However, such findings may be limited to species within the Brassicaceae family due to the presence of the paralogous *CAULIFLOWER* (*CAL*) gene, which arose in this lineage and provides a partially redundant function in the determination of the floral meristem (Lawton-Rauh *et al*., 1999). In these species, the presence of a wild-type copy of *CAL* may partially complement the loss of floral meristem identity in *ap1* mutants and allow floral transitions, and seed production, to occur. In non-Brassicaceae species where *CAL* is not present, mutation of the *AP1* ortholog (*SQUAMOSA* [*SQUA*]) seems to lead to more severe phenotypes with few flowers (Huijser *et al*., 1992) or seeds (Taylor *et al*., 2002), which implies that a CRISPR/Cas-based approach targeting this gene may only be useful in certain oilseed species.

241 SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) transcription factors are
242 known to play a vital role in many plant developmental processes, including juvenile-to-adult
243 and vegetative-to-reproductive transitions, as well as shoot branching (Wang and Wang, 2015).
244 Indeed, a subset of these genes have central functions in a complex flowering regulatory
245 network where they up-regulate the expression of various floral homeotic genes (including
246 *AP1*/*SQUA* homologs), as well as other promoters of the floral transition (Wang et al. 2009a;
247 Wang and Wang, 2015). Due to the roles of several *SPL* genes in shoot branching, their
248 mutation can elicit consequent increases in vegetative biomass (Schwarz *et al*., 2008; Gou *et*
249 *al*., 2017), which could feasibly lead to enhanced seed numbers. In line with this, the
250 CRISPR/Cas9-mediated NHEJ-based disruption of various combinations of soybean *SPL9*
251 homologs resulted in plants with higher leaf, node and branch numbers (Bao e*t al*., 2019).
252 Although seed yield was not assessed in these lines, the results hint at the possibility that
253 increased seed yields could be realized using this strategy. However, many *SPL* genes (or their
254 downstream targets) have undergone neo-functionalization in a number of plant species (e.g.,
255 Martin *et al*., 2009; Preston *et al*., 2012; Van *et al*., 2013), and redundancy between different
256 family members is common. Furthermore, several SPL family members have been shown to
257 function as positive regulators of seed size (Si *et al*., 2016), which could complicate efforts in
258 this area and highlights the importance of elucidating *SPL* networks in a broad range of oilseed
259 species in the near future.
260 The highly conserved *FLOWERING LOCUS T* (*FT*) also plays a central role in the
261 floral transition as an integrator of several major flowering pathways and encodes a florigen
262 that is transported from the leaves to the shoot apical meristem through the phloem to induce
263 floral initiation through the activation of downstream targets, including *AP1* (Jaeger and
264 Wigge, 2007). In addition, *FT* homologs have also been shown to be involved in other

developmental processes, including the outgrowth of lateral shoots (e.g., Hiraoka *et al*., 2012) and the differentiation of storage organs (e.g., Navarro *et al*., 2015). Intriguingly, the CRISPR/Cas9-mediated NHEJ-based mutation of two *FT* homologs in soybean (*GmFT2a* and *GmFT5a*) has been found to lead to substantial photoperiod-dependent delays in flowering compared to wild-type (Cai *et al*., 2018; Cai *et al.*, 2019). In addition, both *GmFT2a* and *GmFT2a*/*GmFT5a* mutants exhibited significant increases in height and node number compared to wild-type plants, while *GmFT2a*/*GmFT5a* double mutants also displayed more than a 250% relative enhancement in seed number per plant compared to wild-type under short days (SD; Cai *et al*., 2019). Since soybean typically initiates flowering under SD, its cultivation has been largely limited to temperate regions. The ability to extend vegetative growth under SD, as appears to be the case in double *GmFT2a*/*GmFT5a* mutants, could provide a step towards the cultivation of this, and other similar species at tropical latitudes (Cai *et al*., 2019).

However, the down-regulation/mutation of *FT* homologs has not always been found to have this same effect. For example, in *B. napus* (which possesses 6 *FT* paralogs), the EMS-mediated mutation of the *BnC6FTa* gene did not impact flowering time; however, the mutation of *BnC6FTb* led to a flowering delay along with a reduction in fertility (Guo *et al*., 2014). Furthermore, while the artificial miRNA-mediated down-regulation of *FT* in *B. juncea* has been shown to increase vegetative biomass and severely delay flowering compared to wild-type, these plants produced only rudimentary siliques with non-viable seeds (Tyagi *et al*., 2018). These data suggest that different *BnFT* paralogs have distinct functions, and that modification of their expression using CRISPR/Cas must be carefully assessed for beneficial agronomical outcomes among species.

B. Alteration in seed cell proliferation

Another approach to increase seed yield includes the modulation of seed size, which is a complex trait involving interactions among the embryo, endosperm, seed coat and parent plant. Various pathways are known to contribute to seed size control (Li *et al*., 2019a), and several negative regulators associated with seed cell proliferation or expansion have been identified to date, although research is sparse in oilseed crop species. For example, *BIG SEEDS (BS)* (also known as *PEAPOD* [*PPD*]) encodes a plant-specific member of the TIFY transcription factor family. In legumes, this gene has been shown to negatively regulate primary cell proliferation, at least in part through the repression of *GROWTH REGULATING FACTOR 5* (*GRF5*) and *GRF-INTERACTING FACTOR 1* (*GIF1*) genes (Ge *et al*., 2016). In line with this, the mutation of *BS* homologs in the legumes *Medicago truncatula* (*BS1*) and blackgram (*Vigna mungo*), as well as the simultaneous down-regulation of both *BS*/*PPD* homologs in soybean, resulted in plants with increased organ size, leading to leaves, pods and seeds that were dramatically greater in size and weight compared to wild-type. This enlargement was due to an increase in cell number rather than cell size (Ge *et al*., 2016; Naito *et al*., 2017). However, where it was assessed, seed number per plant was found to be reduced in these lines, and total seed weight per plant tended to not be altered, or was reduced, compared to wild-type (Naito *et al*., 2017).

CRISPR/Cas9-mediated NHEJ double disruption of these same two genes in soybean was found to lead to two separate phenotypic classes, depending on whether all edits involved frameshift mutations or if one comprised an in-frame deletion, which could theoretically allow at least partial functionality of one homolog (Kanazashi *et al*., 2018). In the case of double frameshift mutations, plants were severely affected in terms of leaf development and very few seeds were produced. However, when one of the mutations was in-frame, plants produced large pods and seeds that were reminiscent of those noted previously with the down-regulation of

these genes (Kanazashi *et al*., 2018). While these plants were also found to produce fewer seeds per plant than wild-type, seed yields were not assessed, and it remains to be determined whether the mutation of only one of two *BS*/*PPD* homologs could provide superior results. Furthermore, the redundant *PPD1* and *PPD2* from Arabidopsis have a slightly different function than their legume counterparts, repressing the asymmetric division of meristemoids and regulating the size and shape of leaves and siliques, but not seeds (White, 2006; Gonzalez *et al*., 2015; Ge *et al*., 2016). Therefore, it may be that this approach, if successful, will be limited to leguminous oilseeds, such as soybean and peanut.

The enhancement of hexose (glucose and fructose) to sucrose ratios in seeds can also have a profound effect on seed yield by stimulating cell proliferation and/or expansion (Weber *et al*., 1996; Wang and Ruan, 2013). In higher plants, cell wall, cytoplasmic and vacuolar invertases are known to play a critical role in determining hexose to sucrose ratios due to their function in the hydrolysis of sucrose into hexoses. This function is highly important in plants, and these proteins are essential for many processes, including sugar metabolism and signaling, development, cell division and differentiation, senescence, abiotic and biotic stress responses, and source-sink interactions (Weber *et al*., 1996; Sturm, 1999; Essmann *et al*., 2008; Jin *et al*., 2009; Sun *et al*., 2014). Correspondingly, the over-expression of these genes has been found to elicit improvements in traits such as enhanced pathogen resistance (Sun *et al*., 2014), augmented salinity and cold tolerance (Fukushima *et al*., 2001; Qian *et al*., 2018), increased water use efficiency and drought resilience (Albacete *et al*., 2014), and elevated grain yield (Wang *et al*., 2008; Li *et al*., 2013).

Plant invertases are regulated not only at the transcriptional level, but also post-transcriptionally through the repressive role of invertase inhibitor proteins (e.g., Jin *et al*., 2009; Qin *et al*., 2016). It follows then that the down-regulation or mutation of genes encoding

337 invertase inhibitors would provide similar phenotypes as those seen in plants over-expressing
338 invertases. Indeed, the constitutive RNAi-mediated silencing of a putative tomato (*Solanum*
339 *lycopersicum*) invertase inhibitor was found to increase cell wall invertase activity, leading to
340 prolonged leaf life span and enhanced fruit hexose levels, as well as elevated seed protein
341 content and seed weight (Jin *et al*., 2009). While relatively little research has been carried out
342 with regards to the function of these genes in oilseed crop species as of yet, the constitutive
343 RNAi-mediated silencing of the *GmCIF1* cell wall invertase inhibitor gene in soybean has
344 similarly been found to increase the activity of cell wall invertases, and the resulting plants
345 displayed improvements in seed weight and protein content compared to wild-type (Tang *et*
346 *al*., 2017). These results suggest that such genes may be ideal targets for NHEJ-mediated
347 mutation via CRISPR/Cas as a means of achieving oilseed yield gains. Temperature-related
348 stress tolerance has also been found to be enhanced in tomato plants with down-regulated
349 invertase inhibitor genes (e.g., Liu *et al*., 2016; Xu *et al*., 2017b), which could lend even
350 further advantages to this approach. However, the RNAi-mediated down-regulation of an
351 invertase inhibitor gene has also been found to lead to reductions in drought tolerance in sweet
352 potato (*Ipomoea batatas*; Yang *et al*., 2020), which indicates that differences may exist among
353 plant species or types of stress, and care will need to be taken to thoroughly assess such
354 parameters in modified lines.

355 *APETALA 2* (*AP2*), which encodes a well-known floral homeotic gene that, like *AP1*, is
356 involved in the establishment of the floral meristem and the specification of floral organ
357 identity, has also been found to play a role in seed development (Jofuku *et al*., 1994). As is the
358 case with *ap1* mutants, plants with mutations in *AP2* produce flowers with homeotic
359 conversions of floral organs (mainly sepals and petals), and in at least certain cases enhanced
360 seed yield (Jofuku *et al*., 1994; Ohto *et al*., 2005). However, despite the similar functional

roles of *AP1* and *AP2* in floral development, the effect of their mutation on vegetative and seed characteristics differs quite substantially. In the case of Arabidopsis *ap2* mutants, alterations in plant architecture are not evident and yield increases are the result of enlarged seeds rather than elevations in seed numbers (Jofuku *et al*., 1994). These seeds also display an increased ratio of hexose to sucrose, which is known to promote cell division and thus may be at least partly responsible for increased seed size (Ohto *et al*., 2005; Ohto *et al.*, 2009). However, mature *ap2* mutant embryos display elevations not only in cell number, but also cell size, and additionally exhibit enhanced accumulation of storage proteins and lipids (Ohto *et al*., 2005; Ohto *et al.*, 2009), which suggests that other mechanisms are also involved, such as an extended period of seed development. In any case, it is possible that the targeting of *AP2* homologs using NHEJ-mediated CRISPR/Cas might have the potential to not only boost oilseed yields, but also improve seed quality.

An alternative approach to boost seed size would be to target genes encoding products that function in seed carbon allocation. One example of such a gene is that encoding ADP-glucose pyrophosphorylase (AGPase), which is a key enzyme of the starch biosynthetic pathway and tends to elicit increases in seed size when down-regulated/mutated in oilseed species. For example, when the *CsAPS* gene encoding the AGPase small subunit was down-regulated in camelina using seed-specific RNAi, the resulting lines exhibited moderate decreases in seed starch accumulation, along with increases in soluble sugar and protein content, as well as enhanced seed size and weight, without any concomitant alterations in seed number, seed oil content or fatty acid composition (Na *et al*., 2018). Increased seed size was found to be the result of larger cells in the seed coats and embryos, and germination was not impacted in these lines. The findings were consistent in both the greenhouse and field, and while this certainly suggests that yields would be improved, this has yet to be assessed in large

385 scale field trials. In contrast to these findings, however, the embryo-specific antisense
386 repression of an *APS* homolog in *B. napus* did not lead to increased seed size or weight
387 (Vigeolas *et al*., 2004), which may be attributable to the different promoters used to drive
388 transgene expression.

389 While both of these studies involved down-regulation of *AGPase* specifically within
390 seed tissues, it has been shown previously that a partial loss-of-function mutation of a gene
391 encoding a catalytic AGPase large subunit (APL1) in Arabidopsis did not lead to any
392 substantial growth penalties under low nitrogen conditions (Schulze *et al*., 1991); however,
393 significant reductions in plant biomass were noted when plants had access to high levels of
394 nitrogen (Schulze *et al*., 1991). An effect on vegetative growth is not surprising in these lines
395 since starch plays an important role in the changing carbon budget of plants under diurnal
396 conditions; a proportion of photosynthate is often stored as starch in the leaves during the day,
397 which is subsequently remobilized at night as a means of supporting respiration and the export
398 of carbon to sink organs (e.g., Zeeman *et al*., 2007). Similarly, mutation of the *APS1* small
399 subunit in Arabidopsis has been found to result in delayed flowering and growth compared to
400 wild-type when grown under a typical day/night photoperiod (Ventriglia *et al*., 2008).
401 However, these growth penalties do not always appear to be the case with constitutive
402 disruption of AGPase subunits since a null mutation within the upstream region of another
403 Arabidopsis gene encoding a distinct APGase large subunit (APL4), which does not exhibit
404 catalytic activity and instead provides regulatory function within sink tissues, led to significant
405 increases in both root and shoot biomass (Sulmon *et al*., 2011). This latter result indicates that
406 the targeting of genes encoding at least certain AGPase subunits using CRISPR/Cas, which
407 results in plant-wide effects and at present cannot be utilized to generate tissue-specific edits

408 unless a transgene is retained in the plant, may still have the potential to provide benefits in the
409 context of seed yields and thus warrants further exploration.

410 Several other pathways have also been shown to be important for seed size determination
411 and may provide additional prospects for CRISPR/Cas targets in oilseeds in the future. For
412 instance, several genes within the ubiquitin-proteasome pathway, including *SAMBA*, *DA1* and
413 *ENHANCER OF DA1/BIG BROTHER* (*EOD1*/*BB*), have been shown to negatively regulate seed
414 size in Arabidopsis by limiting cell proliferation, and their mutation or down-regulation increases
415 seed size (e.g., Li *et al*., 2008; Eloy *et al*., 2012; Vanhaeren *et al*., 2016). Similarly,
416 phytohormone-related pathways are also known to influence seed growth and size (e.g.,
417 Morinaka *et al*., 2006; Riefler *et al*., 2006; Schruff *et al*., 2006). In terms of oilseed crop species,
418 the mutation of *AUXIN RESPONSE FACTOR18* (*ARF18*), which encodes a repressor of auxin-
419 responsive genes, in *B. napus* leads to increases in seed weight (Liu *et al*., 2015a). However, as
420 of yet, relatively little is known about these pathways in oilseed crop species, and additional
421 research will be required to unravel their roles in seed development.

422

423 **III. Reducing yield losses**

424 While boosting seed yields directly is certainly an important target for oilseed improvement,
425 reducing yield losses that are incurred on a regular basis, both pre- and post-harvest, will also be
426 of paramount importance for meeting seed oil demand. The enhancement of pod/silique
427 shattering and herbicide resistance, as well as abiotic and biotic stress tolerance, have been long-
428 standing targets for oilseed breeders for many years (Figure 2). However, these traits are now
429 also beginning to gain attention in the context of CRISPR/Cas-mediated oilseed improvement.

430

431 ***A. Minimization of seed shattering***

Although seed shattering, which refers to pod/silique shattering in legumes and members of the Brassicaceae family, is essential for propagation in many wild plant species, this trait is one of the most critical yield-reducing factors in cultivated seed crops (Funatsuki *et al*., 2014; Steponavičius *et al*., 2019; Tsujimura *et al*., 2019). In canola, it has been estimated that seed yield losses due to seed shattering are typically in the range of 5 to 10%; however, over 40% of the total harvest can be lost as a result of seed shattering in seasons with adverse weather conditions that delay harvesting (Gan *et al*., 2016). In addition to the reduction in economic return sustained by such losses, shattered pods/siliques also increase production costs in subsequent years since the inadvertently dispersed seeds can lead to recurring growth as a weed (Gan *et al*., 2008). As such, enhancing shatter resistance has become a priority in the breeding of certain oilseed crops as a means of maintaining seed yield and boosting profitability. Since differences exist among the shattering mechanisms of different plant groups and our understanding of the precise mechanisms underlying these processes is still incomplete, furthering research in this area will benefit our ability to achieve this goal in the future.

At present, the vast majority of research focusing on the elucidation of molecular mechanisms driving pod/silique shattering has been carried out in Arabidopsis, where intricate regulatory networks involving multiple transcription factors and phytohormones have been unraveled that appear to be conserved in other members of the Brassicaceae. In these species, silique shattering commences with the degradation and separation of cell walls along the length of a layer of cells termed the dehiscence zone (Meakin and Roberts, 1990). The redundant *SHATTERPROOF 1* (*SHP1*) and *2* (*SHP2*), which encode MADS-domain transcription factors, function at the top of the genetic cascade controlling the development of the dehiscence zone in siliques (Lewis *et al.,* 2006). Intriguingly, the constitutive RNAi-mediated silencing of *SHATTERPROOF* (*SHP*) alleles in *B. napus* has been found to lead to the production of

indehiscent siliques with no other obvious morphological abnormalities compared to wild-type, although quantitative measurements of important characteristics such as seed yield were not assessed in this study (Kord *et al*., 2015).

INDEHISCENT (*IND*) and *ALCATRAZ* (*ALC*), which encode basic helix-loop-helix (bHLH) transcription factors and are termed valve margin identity genes, are positively regulated by *SHP1*/*2* and also function in the control of silique dehiscence (Rajani and Sundaresan, 2001; Liljegren *et al*., 2004). *IND* directs the differentiation of lignified and separation layers, which together make up the dehiscence zone (Liljegren *et al*., 2004), whereas *ALC* is required only for the formation of the separation layer (Rajani and Sundaresan, 2001). In Arabidopsis, the mutation of *IND* or *ALC* lead to a lack of valve margin formation, resulting in indehiscent or partially indehiscent siliques (e.g., Rajani and Sundaresan, 2001; Liljegren *et al*., 2004), making them ideal candidates for CRISPR-mediated modulation in the breeding of shatter-resistant Brassicaceae species. Indeed, the CRISPR/Cas9-mediated NHEJ-based homozygous knock-out of the *BnA03.IND* gene leads to increased shatter resistance in *B. napus* compared to wild-type controls. However, the CRISPR/Cas-mediated mutation of the *BnC03.IND* paralog did not have the same effect, and double mutation of both *IND* paralogs resulted in severe defects in silique morphology (Zhai *et al*., 2019). In contrast, the CRISPR/Cas9-mediated NHEJ-based double knock-out of both *BnALC* homologues in *B. napus* was found to either enhance shatter resistance only in siliques longer than 5 cm (Braatz *et al*., 2018) or have no obvious effect on shatter resistance compared to wild-type (Zhai *et al*., 2019), which suggests that this particular approach may have limited applicability in terms of increasing pod shatter resistance (Braatz *et al*., 2018).

Gibberellin 3-oxidase 1 (GA3ox1) catalyzes the final step in the biosynthesis of bioactive gibberellins (Talon *et al*., 1990), which are involved in the regulation of plant growth through their effects on cell division and elongation (Yamaguchi *et al*., 2008). The expression of *Ga3ox1*

480 is directly up-regulated by *IND* in tissues such as valve margins, and its mutation tends to lead to
481 partially indehiscent siliques due to defects in the separation layer (Talon *et al*., 1990; Arnaud *et*
482 *al*., 2010; Stephenson *et al*., 2019). In line with this, the CRISPR/Cas9-mediated NHEJ-based
483 knock-out of one of two *GA3ox1* paralogues in *B. oleracea* led to the production of siliques with
484 defects in silique valve margin development, which suggests that these plants would exhibit at
485 least some level of shatter resistance, although this was not assessed in this study (Lawrenson *et*
486 *al*., 2015). Moreover, the edited plants also displayed a semi-dwarf phenotype, as has often been
487 observed previously in Arabidopsis plants bearing mutations in *GA3ox1* (e.g., Talon *et al*.,
488 1990). While it is currently unknown how this would affect agronomic performance, many crop
489 species exhibiting dwarfism as a result of a reduction in the production of, or insensitivity to,
490 gibberellins have been found previously to display decreased lodging and/or increased seed
491 yields (e.g., Muangprom *et al*., 2005; Zhou *et al*., 2012), and such mutations were important
492 contributors to the 'Green Revolution' promoted in large part by the work of Norman Borlaug
493 (Peng *et al*., 1999; Hedden and Sponsel, 2015). Taken together, this suggests that this approach
494 could provide a promising means of enhancing the productivity of oilseed species in the future.
495
496 ***B. Engineering herbicide tolerance***
497 Weeds invariably need to be managed in cropping systems to prevent competition with crop
498 plants for various resources including sunlight, water and nutrients (Sedeek *et al*., 2019). Their
499 unhindered growth not only substantially reduces the yield of oilseed crops, but also
500 contaminates harvested product, which can be problematic (Asaduzzaman *et al*., 2020). As a
501 means of mitigating these losses, transgenic herbicide-tolerant oilseed varieties have become a
502 mainstay of crop production (Bonny, 2008; Schütte *et al*., 2017), with genetically engineered
503 herbicide-tolerant soybean and cotton, for example, making up more than 90% of their respective

crop acreages in the United States (United States Department of Agriculture Economic Research Service, 2019). The well-known Roundup Ready® trait is one of the most commonly used in terms of herbicide tolerance, and occurs through the genomic insertion of a transgenic cassette including a sequence encoding a glyphosate-insensitive form of 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS) from *Agrobacterium* sp. strain CP4. This renders these plants resistant to the broad-spectrum herbicide glyphosate, and allows its application for weed control on a regular basis (Funke *et al*., 2006). Several other transgenic oilseed varieties have also been developed with resistance to different herbicides, including glufosinate, dicamba and 2,4-dichorophenoxyacetic acid (Nandula, 2019). However, in all cases, they face market and regulatory limitations due to the presence of a transgene, which has provided an impetus to find alternative routes for achieving herbicide tolerance in less widely grown oilseed crops. While several non-transgenic herbicide tolerance traits (e.g., sulfonylurea, imidazolinone and triazine tolerance) have been developed through induced mutagenesis, tissue culture or spontaneous mutations, many of the resulting plants have displayed only modest improvements in agronomic performance overall (e.g., Nandula, 2019; Asaduzzaman *et al*., 2020).

While glyphosate resistance has typically been achieved through the insertion of a glyphosate insensitive *EPSPS* gene of bacterial origin, the precise mutation of endogenous *EPSPS* genes can also elicit the same effect. Normally, the EPSPS enzyme is involved in the biosynthesis of essential aromatic amino acids, but its activity is inhibited by glyphosate, which ultimately leads to plant death (Schönbrunn *et al*., 2001). In flax (*Linum usitatissimum*), two paralogous *EPSPS* genes were edited simultaneously through the transient introduction of a single-stranded oligonucleotide (ssODN) in combination with CRISPR/Cas9, leading to glyphosate tolerance in the resulting plants (Sauer *et al*., 2016). While this technology requires the use of a chemically synthesized oligonucleotide (~150 nucleotides in length), which acts as a

528 DNA template during the editing process and allows precise nucleotide edits to be made at the
529 site of the Cas9-induced double strand break, the resulting edit only consisted of two nucleotide
530 substitutions compared to the endogenous sequence. More specifically, this strategy resulted in
531 the conversion of T178I and P182A; alterations that rendered the enzyme insensitive to
532 glyphosate (Sauer *et al*., 2016).

533 Acetolactate synthase (ALS) is another key metabolic enzyme in the biosynthesis of amino
534 acids, but unlike *EPSPS*, it is involved in the production of branched-chain amino acids such as
535 valine, leucine and isoleucine. Its activity is inhibited by certain classes of herbicides such as
536 sulfonylureas and imidazolinones (Lee et al., 1988), and genome editing techniques such as
537 oligonucleotide-directed mutagenesis, zinc finger nucleases (ZFN), transcription activator-like
538 effector nucleases (TALEN) and CRISPR/Cas9 have been employed in various crops to
539 produce precise alterations in this gene to establish herbicide tolerance to date (e.g., Gocal *et*
540 *al*., 2015; Sun *et al*., 2016; Songstad *et al.*, 2017; Tian *et al*., 2018). For example, CRISPR-
541 mediated base-editing has been used to precisely edit the *ALS1* gene in *B. napus* to elicit a
542 P197S mutation and consequent herbicide tolerance (Wu *et al*., 2020a), and a sulfonylurea-
543 resistant canola variety achieved using ssODN-mediated mutagenesis is already on the market
544 in the US and Canada (SU-canola; Cibus). In addition, HDR-mediated CRISPR/Cas has been
545 used to replace the endogenous *ALS* gene in soybean with a homologous 1,084-bp DNA
546 fragment containing three silent nucleotide substitutions that led to a P178S mutation that
547 should theoretically result in herbicide tolerance; however, resistance was not assessed in this
548 study (Li *et al*., 2015).

549 While many herbicide-tolerant transgenic and conventionally bred oilseed cultivars are
550 already on the market, weed resistance to the various herbicides currently in use is becoming
551 problematic (e.g., Heap and Duke, 2017; Perotti *et al*., 2020). This has led to the stacking of

552 multiple herbicide tolerance traits in many varieties over recent years, and the use of herbicide
553 rotation/mixtures as one component of an integrated crop management approach to mitigate this
554 issue. However, with the hope that new herbicides with novel sites of action will be developed,
555 and currently underutilized oilseed species gain popularity for cultivation, the ability to rapidly
556 and precisely edit plant genes to elicit herbicide tolerance could prove to be extremely valuable.
557 Since this typically requires the generation of specific nucleotide substitutions within a target
558 gene, newer CRISPR/Cas technologies, such as base- and prime-editing, will likely play a
559 central role in such endeavours in the future.
560
561 ***C. Enhancement of abiotic stress tolerance***
562 Environmental stresses such as drought, salinity, and waterlogging, as well as low or elevated
563 temperatures, are major factors limiting the growth, development, seed quality and overall
564 productivity of oilseed crops (Boem *et al*., 1996; Purty *et al*., 2008; Singer *et al*., 2016; Elferjani
565 and Soolanayakanahally, 2018). For example, severe soil water deficits during flowering and pod
566 setting stages in soybean have been found to lead to a 61% reduction in total leaf area per plant, a
567 67% decrease in aboveground biomass, and up to 82% seed yield losses (Wei *et al*., 2018).
568 Unfortunately, these types of stresses are becoming more frequent and their intensity is
569 escalating in many parts of the world due to climate change (AghaKouchak *et al*., 2020), which
570 means that oilseed yield losses resulting from abiotic challenges are likely to worsen in coming
571 years.
572 Although a comprehensive understanding of the cascade of physiological and molecular
573 events that takes place upon exposure to these types of stresses is still lacking, our knowledge in
574 this area has increased markedly in recent years (e.g., Wang *et al*., 2019b; de Souza *et al*., 2020).
575 Abiotic stresses such as drought, hypoxia, salinity and extreme temperatures typically lead to an

influx of calcium (Ca^{2+}) into the cytosol of plant cells, and a surge in the production of reactive oxygen species (ROS), which can damage DNA, RNA proteins and lipids when above a threshold level. The accumulation of Ca^{2+} and ROS then activates various signaling pathways that, along with phytohormones such as abscisic acid (ABA), results in an adaptive and integrated response to maximize survival (Mohanta *et al*., 2018). This response employs numerous mechanisms, including the production of structural or protective proteins, an accumulation of osmoprotectants and enhanced antioxidative capacity. To date, a large number of plants over-expressing various miRNAs (e.g., Arshad *et al*., 2017; Ho *et al*., 2019), as well as genes encoding a number of transcription factors (e.g., Hao *et al*., 2011; Zhu *et al*., 2018), antioxidant enzymes (e.g., Saxena *et al*., 2020), proteins involved in the production of molecular antioxidants (e.g., Kim *et al*., 2019) or osmoprotectants (e.g., Sun *et al*., 2019), and proteins that function in phytohormone signaling pathways (e.g., Sahni *et al*., 2016) have been shown to exhibit an enhanced ability to withstand various types of abiotic stress. However, there is a distinct paucity of information regarding negative regulators within abiotic stress response-related pathways, and therefore studies involving CRISPR/Cas-mediated improvement of abiotic stress tolerance remain scarce.

While various phytohormones are believed to play a role in plant stress response, ABA is particularly well-known for the central role that it plays in a plant's ability to cope with osmotic stress. When plant cells are exposed to osmotic stress, *de novo* ABA biosynthesis is up-regulated, which leads to the transcriptional modulation of many genes and promotes stomatal closure to avoid transpiration-related water loss (Nakashima and Yamaguchi-Shinozaki, 2013). The WD40-repeat family protein RECEPTOR FOR ACTIVATED C KINASE 1 (RACK1) is a known negative regulator of ABA responses in plants (Guo *et al*., 2009; Zhang *et al*., 2013), and the constitutive RNAi-mediated down-regulation of a *RACK1* homolog in soybean has been

found to increase drought and salt tolerance compared to wild-type plants (Li *et al*., 2018c; Zheng *et al*., 2019). Under stress conditions, these lines also displayed enhancements in ABA content, elevated antioxidant enzymatic activities and expression levels, and a reduction in the expression levels of genes involved in ROS production compared to wild-type, which likely all contributed to their stress resilience (Li *et al*., 2018c; Zheng *et al*., 2019). A similar improvement in drought and/or salinity tolerance has also been observed in rice *RACK1* RNAi lines (Li *et al*., 2009; Zhang *et al*., 2018b), which suggests that this approach may be applicable across a wide range of plant species. However, there is also evidence that *RACK1* genes may act as positive regulators in the response to some fungal phytopathogens (e.g., Wang *et al*., 2014b; Li *et al*., 2017a); a finding that will warrant further assessments in knock-down and knock-out lines in the future. Furthermore, loss-of-function Arabidopsis *rack1a* mutants, but not *rack1b* or *rack1c* mutants, have been found to display severe morphological defects under non-limiting growth conditions (Chen *et al*., 2006a; Guo and Chen, 2008) and exhibit hypersensitivity to salt (Guo *et al*., 2009). While the reasons behind these discrepancies among species have yet to be unraveled, it is clear that the specific paralog chosen for CRISPR/Cas-mediated targeting, as well as gene dosage, may all be important factors for consideration with this approach.

In plants, the post-translational farnesylation of proteins, whereby a farnesyl group is added to a conserved cysteine residue at the carboxy terminus of a protein, allows otherwise hydrophylic proteins to function as peripheral membrane proteins and plays a critical role in many cellular processes, including abiotic stress response (e.g., Jamshed *et al*., 2017). The *FARNESYLTRANSFERASE A* (*FTA*) and *ENHANCED RESPONSE TO ABA1* (*ERA1)* genes encode the α and β subunits of farnesyltransferase, which functions in ABA signaling, and the down-regulation/mutation of both genes has been found to result in ABA hypersensitivity, stomatal closure and reduced transpiration rates (Allen *et al*., 2002; Wang *et al*., 2009b). In *B.*

napus, the drought-inducible down-regulation of both *BnFTA* and *BnERA1*, respectively, has been found to provide yield protection under drought stress at flowering without negatively impacting growth under well-irrigated conditions (Wang *et al*., 2005; Wang *et al*., 2009b). However, this approach may be contingent upon the conditional down-regulation of these genes under drought conditions since the mutation of both genes in Arabidopsis causes pleiotropic defects (Yalovsky *et al*., 2000; Running *et al*., 2004; Daszkowska-Golec *et al*., 2018), increased susceptibility to pathogens (Goritschnig *et al*., 2008) and reduced tolerance to moderate sustained heat stress (Wu *et al*., 2016), which would almost certainly limit agronomic usefulness. Since inducible expression with the CRISPR/Cas system is not possible using transgene-free forms of the technology, it remains to be determined whether this strategy would provide benefits in this area. Several other ABA hypersensitive Arabidopsis mutants, such as *abh1* (Hugouvieux *et al*., 2001), *abo1* (Chen *et al*., 2006b), and *cyp85a2* (Northey *et al*., 2016) have also been shown to exhibit enhancements in drought response; however, very little is known about potential pleiotropic effects, or their roles in other plant species, as of yet.

Another important component of stress response involves the signaling molecule nitric oxide (NO), which accumulates under a wide range of stress conditions and elicits its effect via the prevention of oxidative damage by ROS, modulations in phytohormone signaling, and post-translational modifications of target proteins (Asgher *et al*., 2017; Nabi *et al*., 2019). In addition, NO is also known to stimulate the growth of lateral roots, root hairs and adventitious roots (e.g., Foreman *et al*., 2003; Correa-Aragunde *et al*., 2004; Liao *et al*., 2012), which may also contribute to its function in stress response. The enzymatic activity of arginase (ARG), which catalyzes the production of ornithine and urea from arginine, suppresses the production of nitric oxide by nitric oxide synthase due to competition for their common arginine substrate (Shi *et al*., 2013). In line with this, Arabidopsis *arg* mutant lines exhibit higher levels of NO production,

reduced ROS accumulation under stress conditions, and augmented production of lateral and adventitious roots, as well as improvements in drought, salt and freezing tolerance, compared to wild-type plants (Flores *et al*., 2008; Shi *et al*., 2013). Similarly, the simultaneous NHEJ-based CRISPR/Cas9-mediated mutation of two paralogous *GhARG* genes in allotetraploid cotton resulted in plants with increased NO content and enhanced lateral root production compared to wild-type (Wang *et al*., 2017a). While stress tolerance was not assessed in this study, this strategy certainly holds promise for enhancing resilience to abiotic challenges in oilseed crop species in the future.

The ability of plants to withstand salinity involves several processes above and beyond those typically employed during osmotic stress response, many of which center on the prevention of Na^+ accumulation within cells. For instance, the SALT OVERLY SENSITIVE (SOS) pathway consists of three main components, including the calcium-binding protein SOS3, protein kinase SOS2, and plasma membrane Na+/H+ antiporter SOS1 (Zhu, 2002; Guo *et al*., 2004; Ke *et al*., 2017). Under non-limiting growth conditions, GIGANTEA (GI), which is predominantly associated with photoperiodic control of flowering and is a major component of salt stress adaptation (Ke *et al*., 2017), binds SOS2 and prevents the activation of SOS1. However, under salt stress, GI undergoes proteasomal degradation, which promotes the formation of SO2-SO3 complexes that activate SOS1 via phosphorylation, resulting in the export of Na^+ ions from the cell and aiding in a plant's ability to withstand these conditions (Yoon *et al*., 2018). Consequently, GI has been shown to function as a strong negative regulator of salt stress tolerance in various plant species to date, including members of the Brassicaceae family (Kim *et al*., 2013; Kim *et al*., 2016; Ke *et al*., 2017). For instance, the mutation or constitutive down-regulation of *GI* in both Arabidopsis and *B. rapa* resulted in enhanced resilience to salinity stress compared to wild-type plants (Kim *et al*., 2013; Kim *et al*., 2016). Furthermore, transgene-

672 free delivery of CRISPR/Cas9 RNPs into *B. oleracea* protoplasts was also successfully utilized
673 to simultaneously target two *GI* alleles, but phenotypic assessments have yet to be carried out in
674 these lines (Park *et al*., 2019). Since *GI* is also linked with flowering time, the knock-out/down-
675 regulation of *GI* can lead to delayed flowering (Kim *et al*., 2013; Ke *et al*., 2017), which may or
676 may not be desirable in terms of agronomic performance. However, this is not always the case,
677 and the constitutive down-regulation of *BrGI* in *B. rapa* did not result in any alteration of
678 flowering time, suggesting that gene dosage and/or avoiding loss-of-function mutations may be
679 required for the most beneficial outcomes with CRISPR/Cas editing of this gene in the future.
680 Other potential candidates for the NHEJ-based CRISPR/Cas-mediated improvement of
681 abiotic stress tolerance in oilseed species are particular members of the *STRESS-ASSOCIATED*
682 *PROTEIN* (*SAP*) gene family, which contain A20/AN1 zinc finger domains and are often
683 differentially regulated in response to stress (e.g., Huang *et al*., 2008; Xuan *et al*., 2011; Dixit *et*
684 *al*., 2018). Many studies have found that the over-expression of certain *SAP* genes elicits broad
685 improvements in abiotic stress tolerance in many plant species (e.g., Mukhopadhyay *et al*., 2004;
686 Kanneganti and Gupta, 2008; Dixit *et al*., 2018; Zhang *et al*., 2019), or has distinct effects
687 depending on the type of stress (e.g., Huang *et al*., 2008; Xuan *et al*., 2011), which suggests that
688 members of this gene family play differential roles in stress-signaling pathways. In addition, it
689 appears that at least a small subset of these genes act as negative regulators of both abiotic and
690 biotic stress response (e.g., Sharma *et al*., 2015; Kang *et al*., 2017). For example, the down-
691 regulation of *PagSAP1* in poplar has been found to enhance salinity tolerance through an
692 increase in the accumulation of Ca^{2+} and K^{+}, along with a concomitant decrease in Na^{+}, as well
693 as increased expression of stress response genes including *SOS1* and *SOS3* (Yoon *et al*., 2018).
694 Therefore, while the CRISPR/Cas-mediated editing of a homologous gene in an oilseed species
695 also has the potential to elicit a similar effect, very little is currently known regarding the

functions of these genes in oilseed crop species. Given that there are at least fifty-seven *SAP* genes in *B. napus* (He *et al*., 2019), a substantial amount of research will need to be dedicated towards unraveling their precise roles in stress response.

Another important consideration for the development of climate-smart oilseed cultivars is that the vast majority of studies in which abiotic stress tolerance has been assessed thus far have been based upon the effect of a single form of stress. While prolonged or acute exposure to any single abiotic stress can be enough to devastate oilseed crop yields in the field, several stresses often occur simultaneously in various combinations and at varying levels, which can compound the resulting negative effects (Elferjani and Soolanayakanahally, 2018). The precise molecular effects of such interactions have not been well-studied, and an improved understanding of abiotic stress response mechanisms under complex growing conditions will therefore be of the utmost importance for maximizing our ability to provide oilseed crop improvements using any breeding platform in the future.

D. Improvement of disease resistance

Biotic stress caused by phytopathogens can result in considerable crop yield losses both before and after harvest (Savary *et al*., 2012). Moreover, with impending climate change scenarios, the establishment and long-term survival of existing phytopathogens, as well as the emergence/spread of new and aggressive species, may very well increase in coming years (Chattopadhyay *et al*., 2019; Wu *et al*., 2020b). While conventional breeding has been utilized to develop varieties with improved resistance to certain pathogens, large gaps still remain in terms of controlling a vast number of biotic stressors in a wide range of crop species. Therefore, an ability to develop new oilseed cultivars with improvements in biotic stress resilience will almost

719 certainly be of critical importance for oilseed breeders in terms of meeting growing demand with
720 the added benefit of reducing fungicide and/or pesticide use.

721 The vast majority of breeding attempts to enhance disease resistance in crop species have
722 focused primarily on the introgression or transgenic over-expression of resistance (R) genes
723 (Ercolano *et al*., 2012), which in many cases has led to reductions in disease severity and/or
724 incidence (e.g., Li *et al*., 2019b; Qi *et al*., 2019; Xun *et al*., 2019). Most of these genes encode
725 cell surface or intracellular receptors, which can trigger disease resistance in numerous ways,
726 typically through recognition of the pathogen (Kourelis and van der Hoorn, 2018). Alternatively,
727 resistance to certain phytopathogens can also be achieved through the impairment of susceptible
728 (S) genes, which encode products that phytopathogens require for their initial establishment, as
729 well as their subsequent growth and proliferation (Pavan *et al*., 2010). As such, the disruption of
730 such genes using either conventional or genome editing approaches can break host-pathogen
731 compatibility by impairing pre-penetration (e.g., host recognition, penetration, leaf surface
732 modulation) or post-penetration (e.g., nutrients) processes, and can provide broad-spectrum and
733 durable resistance against bacterial and fungal pathogens (Hernández-Blanco *et al*., 2007; Bai *et*
734 *al*., 2008; Wang *et al*., 2014a).

735 Fungal diseases can have a serious impact on oilseed crop production, and are largely
736 controlled through cultural practices, host plant resistance and the use of chemical fungicide
737 applications. However, fungicides can be costly and pose health risks for growers, achieving
738 appropriate timing can be challenging, and the development of fungicide resistance is becoming
739 increasingly problematic (e.g., Carter *et al*., 2014; Derbyshire and Denton-Giles, 2016). In
740 addition, cultivars offering full resistance to particular pathogens are lacking for many crop
741 species (e.g., Derbyshire and Denton-Giles, 2016). For these reasons, attempts to modulate
742 fungal disease resistance using CRISPR/Cas-based methods have been gaining momentum in

743 recent years, and several strategies have shown great promise (e.g., Wang *et al*., 2016; Wang *et*
744 *al*., 2018). Under mild temperatures and humid conditions, oilseed crops are negatively affected
745 by powdery mildew (PM), which can result in yield losses as high as 20-30% (Uloth *et al*., 2018;
746 Dunn and Gaynor, 2020). Intriguingly, the CRISPR/Cas-mediated NHEJ knock-out of the S
747 gene *MILDEW RESISTANCE LOCUS O* (*MLO*), which confers susceptibility to various PM-
748 causing phytopathogens in a broad range of crop species (Shen *et al*., 2012), has been utilized to
749 successfully enhance resistance to PM in wheat (Wang *et al.* 2014a) and tomato (Nekrasov *et al*.,
750 2017). While little is known about *MLO* homologs in oilseed species as of yet, these genes
751 appear to be highly conserved across a wide range of plant species (Pessina *et al*., 2016), and
752 Arabidopsis *mlo* mutants have also been found to exhibit enhanced PM resistance (Frye *et al*.,
753 2001; Consonni *et al*., 2010; Acevedo-Garcia *et al*., 2017). Therefore, it is likely that the
754 CRISPR/Cas-mediated targeting of *MLO* homologs could also yield beneficial results in oilseed
755 crops.

756 Similarly, the CRISPR/Cas9-mediated NHEJ-based bi-allelic/homozygous disruption of
757 two *Gh14-3-3d* homologs in cotton has been found to lead to improvements in resistance to the
758 fungal pathogen *Verticillium dahliae* (Zhang *et al*., 2018c). Members of the 14-3-3 protein
759 family are involved in a wide range of biological functions in plants, and act by binding
760 numerous other proteins to regulate their degradation, activity, or sub-cellular localization (Paul
761 *et al*., 2005). They are highly conserved across plant species, including *B. napus* (Zhan *et al*.,
762 2010) and soybean (Li and Dhaubhadel, 2011), with at least certain members having been
763 suggested to provide a role in signaling pathways and stress response (Seehaus and Tenhaken,
764 1998; Lapointe *et al*., 2001). Therefore, while it is feasible that similar outcomes could be
765 achieved in other oilseed species using CRISPR/Cas, relatively little is currently known
766 concerning the precise function of the numerous *14-3-3* homologs in oilseed species as of yet,

767 and further research will therefore be a pre-requisite for the successful application of this
768 approach.

769 Genes encoding certain members of the WRKY transcription factor domain family have
770 also been found to play an important role in plant disease resistance, acting as either positive or
771 negative regulators of defense. Indeed, numerous studies have demonstrated improvements in
772 resistance to a wide range of phytopathogens (including both fungal and bacterial diseases) as a
773 result of their over-expression (e.g., Abbruscato *et al*., 2012; Yu *et al*., 2012) or down-
774 regulation/mutation (e.g., Journot-Catalino *et al*., 2006; Li *et al*., 2017b) in a multitude of plant
775 species to date. In line with this, the simultaneous CRISPR/Cas9-mediated NHEJ-based knock-
776 out of three out of four *BnWRKY70* paralogs (two mono-allelic mutations and one bi-allelic
777 mutation) in *B. napus* has been found to lead to a small reduction in the size of *Sclerotinia*
778 *sclerotiorum*-induced lesions on detached leaves compared to wild-type. These differences were
779 significant in two of the four lines assessed, suggesting that the *BnWRKY70*-edited plants may
780 possess at least some enhancement in their resistance to this pathogen (Sun *et al*., 2018). Since *S.*
781 *sclerotiorum* affects virtually all dicotyledonous plant species (Bolton *et al*., 2006) and can have
782 a considerable impact on yield in oilseed crops (e.g., del Rio *et al*., 2007; Peltier *et al*., 2012),
783 additional research in this area is warranted. However, further elucidation of the roles of each
784 family member in oilseed species will likely be necessary for the implementation of such a
785 strategy since at least some *WRKY* genes appear to provide differential effects depending on the
786 particular type of pathogen (Wang *et al*., 2017b) or type of stress (Liu *et al*., 2015b).

787 While little progress has been made to date in oilseed species with respect to the
788 modulation of negative regulators controlling resistance to bacterial or viral diseases, progress is
789 being made in other plant species in these areas that could potentially be applied to oilseed crops
790 in the future. For example, CRISPR/Cas has been applied to Arabidopsis, cassava (*Manihot*

esculenta) and cucumber (*Cucumis sativus*) to disrupt *EUKARYOTIC TRANSLATION INITIATION FACTOR 4E* (*eIF4E*) homologs, which are known to be a major susceptibility factor for RNA viruses (Bastet *et al*., 2017), leading to improved resistance to various potyviruses (Chandrasekaran *et al*., 2016; Pyott *et al*., 2016; Bastet *et al*., 2019; Gomez *et al*., 2019). No obvious morphological defects were observed in the edited lines, and where it was assessed, seed yield was not negatively affected (Bastet *et al*., 2019). Furthermore, the RNAi-mediated down-regulation of *eIF4E* in soybean has also been found to lead to enhancements in potyvirus resistance, suggesting that this approach could be broadly applicable across species (Gao *et al*., 2020).

IV. Conclusions

Crop improvement programs could benefit tremendously from the advent of several CRISPR/Cas-based genome editing tools that offer simple and low-cost options for plant breeding, and provide non-transgenic germplasm. There is an imminent need to develop higher yielding oilseed crops with concomitant reductions in losses associated with seed shattering, weed invasion and environmental challenges in order to fulfill the demand of our ever-increasing population under a changing climate. Although a small number of traits in oilseeds have been modulated using CRISPR/Cas platforms thus far, there is much room for further research in which current approaches are expanded to other oilseed species, or potential target genes identified previously through mutation or RNAi-mediated down-regulation are assessed with CRISPR/Cas.

The adjustment of a number of additional traits also has the potential to contribute to oilseed yield increases, including enhancements in photosynthetic efficiency/capacity, response to agronomic inputs and pest tolerance. However, very little progress has been made as of yet

regarding the identification of negative regulators within these processes that would benefit from NHEJ-mediated disruption. Similarly, proteins that could be improved via a small number of nucleotide substitutions using base- or prime-editing remain scarce, which has hindered efforts thus far. Therefore, attempts to utilize CRISPR/Cas to up-regulate target gene expression, rather than knock-down/knock-down gene function, may be a better option in these instances. This approach could also substantially facilitate gains in other areas, such as abiotic stress tolerance and disease resistance. Although this can be technically challenging to achieve in a manner that would yield non-transgenic germplasm, the disruption of repressor elements within target gene promoters, as has been demonstrated previously in tomato (Rodríguez-Leal *et al*., 2017), or the NHEJ-based mutation of upstream open reading frames within 5' untranslated regions of a target gene, which has been shown to increase translation of the associated mRNA (Zhang *et al*., 2018d), could both provide valuable options in this field. Alternatively, at least in cases where transcriptional silencing of a gene is directed by DNA methylation, the fusion of a catalytically inactive Cas protein (dCas) to either the catalytic domain of the Arabidopsis REPRESSOR OF SILENCING 1 (ROS1) glycosylase or a C-terminal tail that is recognized and bound by a separate module containing the catalytic domain of the human TEN-ELEVEN TRANSLOCATION1 (TET1cd) demethylase could be used to effectively trigger cytosine demethylation at a targeted location. This has been shown to lead to transcriptional up-regulation of the associated gene (Gallego-Bartolome *et al*., 2018; Devesa-Guerra *et al*., 2020), and such epigenetic alterations appear to be heritable, remaining present even once the transgene is segregated out (Gallego-Bartolome *et al*., 2018). While these tools are more challenging to implement than simple NHEJ-based CRISPR/Cas knock-outs elicited through mutations within coding sequences, they have the potential to expand CRISPR/Cas-editing capacity immensely in the future.

Although the use of CRISPR/Cas for oilseed improvement holds great promise for increasing the pace and precision of breeding in coming years, and the vast amount of genomic data for a wide range of species is simplifying such efforts, a major bottleneck remains in the fact that many oilseed species, or agronomically-important genotypes, remain recalcitrant to *in vitro* regeneration, and thus genetic transformation in general (e.g., Maheshwari *et al*., 2011; Sujatha and Tarakeswari, 2019). As such, the development and optimization of genotype-independent transformation protocols for these species will be of the utmost importance for the successful implementation of CRISPR/Cas editing technologies. Furthermore, concerns have also been raised regarding the possibility of off-target effects derived from the use of these tools, and the frequency with which these occur remains unclear. However, while off-target mutations have been found to occur in plants in certain cases (Sun *et al*., 2015), in the vast majority of instances CRISPR/Cas editing has been shown to be highly precise in plants (e.g., Nekrasov *et al*., 2017; Feng *et al*., 2018; Lee *et al*., 2018; Li *et al*., 2019c; Graham *et al*., 2020). In any case, the propensity for off-target effects can be minimized using a variety of approaches, including the careful selection of target sites, the introduction of RNPs (Murovec *et al*., 2018) or the fusion of dCas to the FokI nuclease (Guilinger *et al*., 2014), as well as the use of truncated gRNAs (Fu *et al*., 2014), paired Cas9 nickases with paired gRNAs (Mikami *et al*., 2016), or alternative Cas enzymes (Strohkendl *et al*., 2018).

In addition to their potential use for the improvement of oilseed crop species that are widely grown, CRISPR/Cas also holds promise for furthering the *de novo* domestication of wild or underutilized oilseed species through the targeting of multiple genes known to be involved in the domestication process (McGinn *et al*., 2019). Such a feat has been accomplished in stress-tolerant tomato wild relatives previously (Zsögön *et al*., 2018). While the use and development of CRISPR/Cas-based technologies is just beginning to take off in oilseed species, it is clear that

these highly precise molecular breeding tools have the potential to provide an unprecedented rate of productivity-related improvements in agronomically-valuable oilseed crops, and could thus provide a substantial contribution towards our ability to sustainably meet future demand for oilseed-derived products.

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Declaration of interest

The authors declare that they have no conflict of interest.

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Figure legends:

Figure 1. Main CRISPR/Cas-related approaches for eliciting targeted genome editing and the generation of non-transgenic edited plants. Red indicates nucleotide changes in targeted region, dark purple denotes the PAM. Cas, CRISPR-associated protein; NHEJ, non-homologous end-joining; PAM, protospacer adjacent motif; pegRNA, prime-editing guide RNA; RTase, reverse transcriptase; sgRNA, single guide RNA.

Figure 2. Possible routes, traits and target genes for increasing oilseed crop productivity via CRISPR/Cas-mediated genome editing. *14-3-3*, encodes a member of the 14-3-3 protein family; *APS*, encodes the small subunit of ADP-glucose pyrophosphorylase; *ALC*, *ALCATRAZ*; *ALS*, encodes acetolactate synthase; *AP1*, *APETALA 1*; *AP2*, *APETALA 2*; *ARF18*, *AUXIN RESPONSE FACTOR 18*; *ARG*, encodes arginase; *BS*, *BIG SEEDS*; *CIF1*, encodes a cell wall invertase inhibitor; *CLV1*, *2* and *3*, *CLAVATA* homologs; *DA1*, encodes ubiquitin-activated peptidase; *eIF4E*, *EUKARYOTIC TRANSLATION INITIATION FACTOR 4E*; *EOD1*/*BB*, *ENHANCER OF DA1/BIG BROTHER*; *EPSPS*, encodes 5'enolpyruvylshikimate 3-phosphate synthase; *ERA1*, *ENHANCED RESPONSE TO ABA1*; *FT*, *FLOWERING LOCUS T*; *FTA*, *FARNESYLTRANSFERASE A*; *GA3ox1*, encodes gibberellin 3-oxidase; *GI*, *GIGANTEA*; *IND*, *INDEHISCENT*; *MAX1*, *MORE AXILLARY GROWTH 1*; *MLO*, *MILDEW RESISTANCE LOCUS*

O; *RACK1*, *RECEPTOR FOR ACTIVATED C KINASE 1*; *SAMBA*, negative regulator of the anaphase-promoting complex/cyclosome; *SAP*, *STRESS-ASSOCIATED PROTEIN*; *SHP1*, *2* and *3*, *SHATTERPROOF* homologs; *SPL*, *SQUAMOSA PROMOTER BINDING-LIKE*; *WRKY70*, encodes a member of the WRKY transcription factor family.

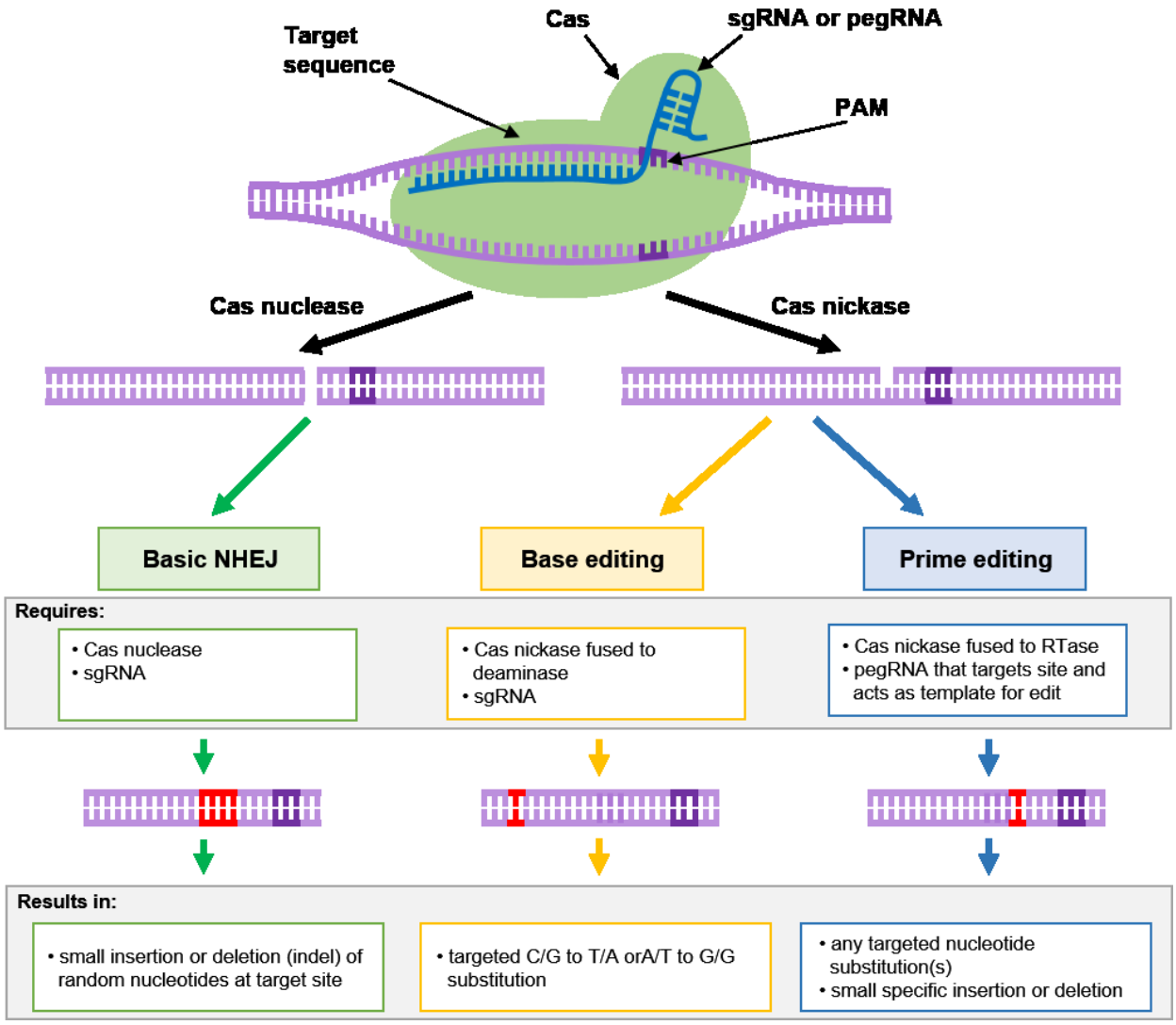
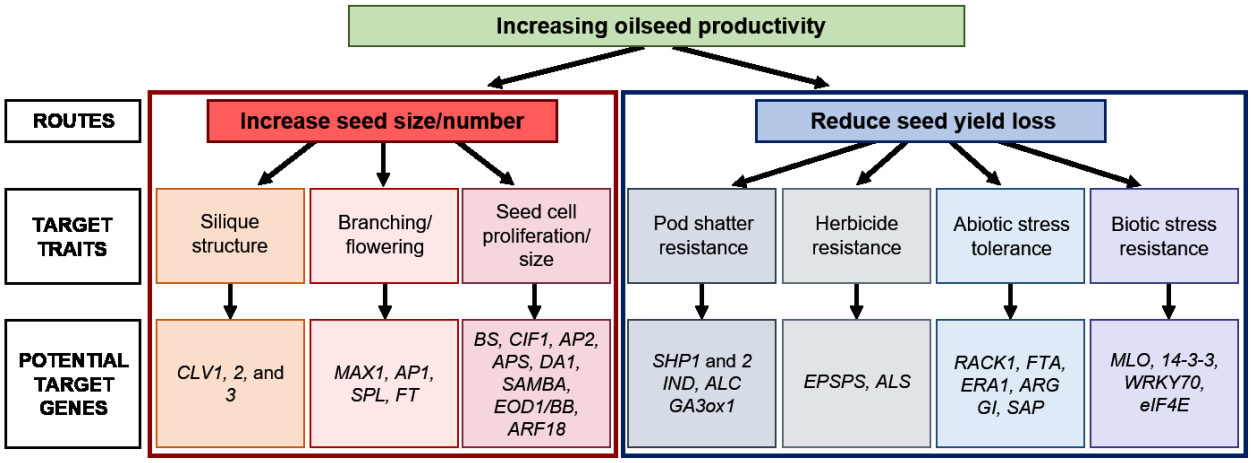

Target sequence
Cas
sgRNA or pegRNA
PAM
Cas nuclease
Cas nickase
Basic NHEJ
Base editing
Prime editing
Requires:
• Cas nuclease
• sgRNA
• Cas nickase fused to deaminase
• sgRNA
• Cas nickase fused to RTase
• pegRNA that targets site and acts as template for edit
Results in:
• small insertion or deletion (indel) of random nucleotides at target site
• targeted C/G to T/A orA/T to G/G substitution
• any targeted nucleotide substitution(s)
• small specific insertion or deletion

Fig 1

2000



2001

2002

2003 **Fig 2**