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SPECIAL ISSUE ON GENOME EDITING

Gene editing in *Brassica napus* for basic research and trait development

Greg F. W. Gocal[1]

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Abstract

The genome of *Brassica napus* L. is the result of several polyploidization events that occurred during the history of *B. napus*. Due to its relatively short domestication history, diversity is relatively limited. An increasing number of loci in this crop's genome have been gene-edited using various technologies and reagent delivery methods for basic research as well as for trait development. New alleles have been developed as edits in single, 2, 4, or more homologous loci in this important oilseed crop. This comprehensive review will summarize new alleles that have been developed as they relate to weed control, flowering, self-incompatibility, plant hormone biology, disease resistance, grain composition, and pod shatter reduction. These new alleles have significantly augmented our understanding of both plant growth and development for basic research as well as for their potential commercial impacts.

Keywords *Brassica napus* · Gene editing · Pod shatter · Herbicide tolerance · Flowering · Hormone signaling · Disease resistance

Introduction

Genomic variation CRISPR/Cas9 has already proven to be a powerful tool for affecting numerous plant traits that have the potential to add diversity to existing organisms while offering significant benefits to plant breeding programs focused on different areas of crop improvement. One such example comes from the work of Rodríguez-Leal *et al*. (2017) who used a gene editing approach to develop a rich source of allelic diversity for breeding in tomato. In this way, gene editing technologies are a logical extension of plant breeding and can be applicable across a wide range of plants (van Eck 2020).

Genome variation is the primary source of phenotypic trait expression in plants. Farmers and plant breeders have harnessed existing genetic diversity using many different methods over thousands of years, beginning with mass selection to identify and select better performing plants, utilizing a better understanding of genetics and inheritance to develop modern plant breeding approaches, and more recently the application of advanced biotechnology tools such as genetic markers, tissue culture, and molecular biology, among other technological advancements. Plants have evolved complex genomes to be able to better adapt to challenges in the environment and evolve as a species. In some cases, the evolution of gene families is characterized by gene duplication resulting from polyploidization, tandem gene duplication, and segmental duplication. The outcomes of all these events are potentially better adapted organisms for specific growing environments.

The source of variation utilized by plant breeders for trait development is in the existing diversity of the target species and its ancestors. Conventional plant breeding methods involve the evaluation and selection of potential parental plant characteristics, with the goal of combining those traits leading to improved performance. Additionally, the breeder will be interested in selecting for traits which may add special characteristics like improved quality or herbicide tolerance to add to the overall yield performance. The process of plant breeding can take several generations of crossing, selfing, and selection to achieve the desired outcome, which can take many years or decades to achieve. The specific length of time depends on the biology of the crop (e.g., length of a typical growth cycle) and the complexity of the trait combinations that are sought. The more complex the trait combination, the longer and more difficult the process. Identifying and selecting an elite combination of genetics in a single variety

✉ Greg F. W. Gocal
ggocal@cibus.com

[1] Cibus US LLC, 6455 Nancy Ridge Drive, San Diego, CA 92121, USA

can take up to 10 yr for row crop species. This presents a significant challenge as our population grows, increasing the need for sustainably produced food. In some cases, the desired trait does not exist in the elite breeding population or is present but very difficult to select because the trait requires several different genes interacting appropriately in order to be expressed. If multiple genes exist to define the desired trait, plant breeders will use several cycles of backcrossing to move the trait into elite genetic backgrounds, although the more complex traits may also lead to increased linkage drag from the donor material. If the desired trait does not exist in the elite material forming the core of the breeding program, laboratory tools such as inter-species crossing, mutagenesis, transgenic development, or gene editing can be used to assist in developing the trait expression of interest or to create new genetic combinations. As will be detailed in this review article focused on *Brassica napus*, several gene editing tools have been used including CRISPR/Cas9 to develop loss of function (LOF) alleles for various loci and base editing to enhance diversity towards developing traits in this important oilseed crop.

***Brassica napus*: a crop for trait development using gene editing** The genus *Brassica* consists of extensively agronomically diverse species. The triangle of U (Figure 1) summarizes the interspecific hybridization events between diploid progenitors *B. rapa* (syn. campestris; AA), *B. nigra* (BB), and *B. oleracea* (CC) resulting in the allotetraploids, *B. juncea* (AABB), *B. carinata* (BBCC), and *B. napus* (AACC) (Nagaharu *et al.* 1935; Snowdon *et al.* 2002). The natural crossing of *B. rapa* (AA; n=18) by *B. oleracea* (CC; n=20) that led to *B. napus* (AACC; n=38) occurred approximately 7,500 yr ago (Chalhoub *et al.* 2014). *B. napus* is the result of a process of 72 genome multiplication events (×3 [γ] ×2 [α] ×2 [β] ×3 ×2) since the origin of angiosperms. Most recently, this includes the whole genome triplication event characteristic of the *Brassicaceae* tribe and a duplication in the coming together of *B. rapa* and *B. oleracea* (Chalhoub *et al.* 2014). Oilseed canola include *B. rapa*, *B. napus*, and *B. juncea*, varieties with international standards for erucic acid and glucosinolate contents (Sharafi *et al.* 2015). *B. napus*—canola, as it is known in North America, or oilseed rape (OSR), as it is known in Europe—is cultivated mainly for its oil-rich seed, the third largest source of vegetable oil in the world (https://apps.fas.usda.gov/psdonline/circulars/oilseeds.pdf). Its oil can also be used for industrial purposes and the meal, as a byproduct of the crush, is used as animal feed—principally in poultry and dairy operations. Canola is the second most profitable oilseed row crop, ranking only behind soybean in production and value (Foreign Agricultural Service/USDA, 2021). There are two types of OSR, spring (SOSR) and winter (WOSR). SOSR, as its name indicates, is planted in the spring and harvested in the fall. By comparison, WOSR is planted in the late summer or fall,

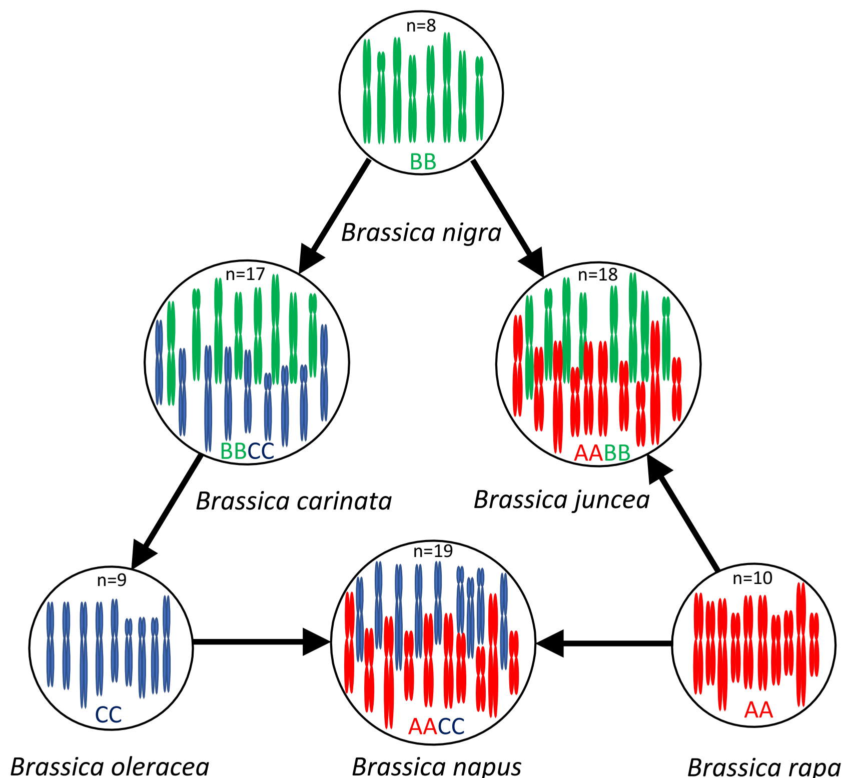

Figure 1 The triangle of U describes the origin of the allotetraploid species *B. juncea* (AABB), *B. carinata* (BBCC), and *B. napus* (AACC) as a result of interspecific hybridization events between the diploid progenitors *B. rapa* (AA), *B. nigra* (BB), and *B. oleracea* (CC).

germinates with the seedling establishing before winter sets in, and then in the spring continues its growth culminating with flowering, seed set, and harvest in the following summer. SOSR types are grown in the Americas, Australia, and northern Europe, whereas WOSR is primarily grown in Europe and China. This review will focus on gene editing of OSR.

Trait development opportunities While gene editing technologies have seemingly unlimited potential to develop novel traits, one key element of deploying these tools is to have a deep understanding of potentially complex trait architectures. Knowing what to edit is a challenge for trait developers to be able to adapt gene editing into a tool for breeders to deploy on a routine basis. Due to significant recent advances in sequencing technologies, computational biology, and bioinformatics, the fields of functional genomics and population genetics have opened up new avenues enabling the understanding of underlying trait architectures thus expanding the possibilities for gene editing. In many cases, basic research in model systems can also help elucidate the effects of individual gene functions, even for complex gene families, however, applying that basic knowledge to crops of interest is required to fully validate that the targeted gene edits translate from model systems into crops. Based on the peer-reviewed literature, the sections that follow discuss the current view of gene editing for basic research and for trait development in *Brassica napus* in several impact areas.

Herbicide tolerance In the developed world, most growers deploy weed control systems to maximize yield by reducing or preventing weed competition for space, nutrients, water, and light. In the peer-reviewed scientific literature, there are many mutations described for various gene targets that confer resistance to a variety of herbicide chemistries. Amino acid substitutions at position P197 in *ACETOHYDROXY ACID SYNTHASE* (*AHAS*) paralogs, based on amino acid positions of the *Arabidopsis thaliana* protein (At3g48560), confer resistance to AHAS-inhibiting herbicides such as tribenuron-methyl in various plants (Chen *et al*. 2017). Editing of homologs of *AHAS* genes has been performed in several plant species, including *Arabidopsis*, corn, rice, and tobacco (Beetham *et al*. 1999; Zhu *et al*. 1999; Zhu *et al*. 2000; Kochevenko and Willmitzer 2003; Okuzaki and Toriyama 2004). More recently, several groups have also performed AHAS editing experiments using a base editing approach (Mishra *et al*. 2020; Wu *et al*. 2020a; Cheng *et al*. 2021). This methodology has emerged as an alternative tool to make nucleotide substitutions in a programmable manner without requiring a double strand break and has been deployed in a variety of plants including *Arabidopsis*, cotton, maize, rice, tomato, and wheat (Komor *et al*. 2016; Mao *et al*. 2019; Mishra *et al*. 2020). Cytidine base editor (CBE) and adenine base editor (ABE) are two versions currently in use, with ABEs developed through directed evolution of *E. coli* TadA (Gaudelli *et al*. 2017; Mishra *et al*. 2020). The methodology involves a Cas9 nickase (nCas9) or catalytically dead Cas protein (dCas9) fused to cytidine deaminase or adenosine deaminase that catalyze the deamination of cytosine (C) to uracil (U) or adenine to inosine (I), respectively. Uracil is recognized as thymine (T) and inosine recognized as guanine (G) during replication allowing for base changes in daughter cells. Wu *et al*. (2020a) used a CRISPR/Cas9-mediated CBE approach to attempt to edit three of the *AHAS* gene targets in canola, *BnAHAS1* (BnaC01g25380D), *BnAHAS2*, and *BnAHAS3* (BnaA01g20380D). A CBE construct containing the rat cytidine-deaminase (rAPOBEC1) was transformed into canola cv. J9712 hypocotyls using *Agrobacterium tumefaciens* (Wu *et al*. 2020a), generating 230 independent T_0 plants. Of these putative transformants, 217 (94%) were PCR positive using vector-specific primers. Of these, amplicons within the target region of each *BnAHAS* locus were sequenced revealing no lines had the expected transitions in *BnAHAS2* or *BnAHAS3* and 7 plants had edits in the *BnAHAS1* gene target. The edited *BnAHAS1* alleles consisted of 4 lines showing an intended C to T transition at amino acid position 197 with the remaining 3 lines displaying (11, 11, and 12 bp) deletions. Three of the 4 lines with edits in BnAHAS1 had an amino acid substitution P197S (CCT to TCT) with the fourth line having an amino acid substitution P197F (CCT to TTT). When sprayed with the AHAS inhibiting herbicide tribenuron, plants homozygous for the P197S allele were more tolerant than those heterozygous for this allele with the WT being severely damaged (Wu *et al*. 2020b). Amino acid substitutions at this position, observed in *Arabidopsis* and in a number of several weed species, confer resistance to various AHAS-inhibiting herbicides including tribenuron-methyl (Chen *et al*. 2017).

Cheng *et al*. (2021) also used a CBE that combined CRISPR-Cas9 nickase with a different cytidine deaminase APOBEC1 and uracil glycosylase inhibitor to introduce targeted C-to-T transition mutations near the PAM site of *BnAHAS1* (BnaC01g25380D) and *BnAHAS3* (BnaA01g20380D). Based on the method of Liu *et al*. (2014), the authors accomplished this by hypocotyl transformation of the semi-winter OSR cv. Zhongshuang 6 using *Agrobacterium tumefaciens*. Of the 38 transgenic plants targeting these loci, 9 plants had edits in the BnaA01g20380D locus, and 8 plants had edits in the BnaC01g25380D locus. Of the mutations that were shown, a CCT to TTT codon substitution encoding a P197F amino acid substitution was observed in 2 plants for each of the *BnAHAS1* and *BnAHAS3* loci. Plants with homozygous mutations in both *BnAHAS1* and *BnAHAS3* were more tolerant to tribenuron than those with homozygous mutations in each AHAS locus (Cheng *et al*. 2021). While base editing methods have been shown effective in certain scope, substitutions arise in a narrow mutagenesis window of ~4 to 9 bases, in which multiple transitions may occur depending on the sequence

context (Mishra *et al.* 2020). This can result in multiple inaccurate editing outcomes.

Flowering, plant hormones, and architecture-Flowering To coordinate the vegetative to floral transition, plants need to achieve the developmental competence to respond to environmental factors such as day length (photoperiod), and winter temperature (vernalization), as well as integrate endogenous signals including phytohormones (e.g., gibberellins) and sugars. In higher plants, flowering time and floral meristem identity are regulated by members of the phosphatidylethanolamine-binding protein (PEBP) family including *FLOWERING LOCUS T* (*FT*), a floral activator, and *TERMINAL FLOWER 1* (*TFL1*), a floral repressor (Kardailsky *et al.* 1999; Kim *et al.* 2013). Five *BnaTFL1* gene copies were identified in the genome of canola (Sriboon *et al.* 2020). Previously, mutations in *BnaA10.TFL1* were shown not to have a significant effect on flowering time (Guo *et al.* 2014). Sriboon *et al.* (2020) used hypocotyl transformation of cv. Westar using *Agrobacterium tumefaciens* with the CRISPR/Cas9 vector having guide RNA designed to target LOF mutations in the 5 *BnaTFL1* loci (BnaA02G0014100ZS, BnaA10G0288700ZS, BnaC02G0013900ZS, BnaC03G0016500ZS, and BnaC09G0608000ZS). Using the Zhang *et al.* (2015) protocol, callus lines of canola cv. Westar were transformed with two sets of constructs, each containing two gRNAs. Of 231 transgenic lines isolated, 100 were CRISPR/Cas9 PCR positive with 18 having at least one edited BnaTFL1 locus (Sriboon *et al.* 2020). The BnaC03.TFL1 biallelic LOF mutant exhibited an early flowering phenotype, while the other LOF mutant lines for the other gene copies had similar flowering times as the WT (Sriboon *et al.* 2020). Furthermore, LOF mutants of individual Bnatfl1 loci displayed altered plant architecture including significant reductions in plant height, branch initiation height, branch number, silique number, number of seeds per silique, and number of siliques on the main inflorescence (Sriboon *et al.* 2020). Together, as Sriboon *et al.* (2020) suggest, these results indicate functional divergence within this gene family in canola.

Also implicated in the vegetative to floral transition, the *Arabidopsis sdg8-2* mutant flowers early because the gene expression of both the MADS-box transcription factor *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) and *FT* are derepressed (Jiang *et al.* 2018). In *Arabidopsis*, histone H3 lysine 4 trimethylation (H3K4me3) and H3K36me3 of core histone tails are required for the activation of *FLOWERING LOCUS C* (*FLC*) expression (Pien *et al.* 2008; Xu *et al.* 2008). When the expression of *FLC* is repressed following sufficient vernalization, it acts upstream of the integrators of the vegetative to floral transition—*SOC* and *FT* (described above).

While in canola it is not unexpected to have one to many paralogs of a target gene arising from each of the A and C genomes, to date within the genome of sequenced plants, the *SET DOMAIN GROUP 8* (*SDG8*) histone lysine methyltransferase had only been observed as a single-copy gene. This presented an opportunity to understand functional divergence with the *BnaA07.SDG8* gene having a single newly identified NNC domain with two adjacent domains in the *BnaC06.SDG8* gene (Jiang *et al.* 2018). Jiang *et al.* (2018) transformed *B. napus* cotyledons of cv. XiangYou15 using *Agrobacterium tumefaciens* with a CRISPR/Cas9 vector designed to make LOF mutations in the 2 *BnaSDG8* genes (*BnaA07.SDG8* (BnaA07g33460D) and *BnaC06.SDG8* (BnaC06g38010D)). Of 24 independent transformants obtained, 20 were PCR positive for the sgRNA-Cas9 cassette of which 3 lines displayed putative LOF alleles in the BnSDG8 loci. One line had biallelic mutations in each BnaSDG8 locus. A locus with one of those alleles remaining in frame having a 3-bp deletion, the second line had biallelic mutations in *BnaC06.SDG8*, and the third line termed Bnasdg8-1 was homozygous for LOF alleles in each of *BnaA07.SDG8* and *BnaC06.SDG8* (Jiang *et al.* 2018). Compared to the WT control, flowering time of null segregants lacking the transgene showed that Bnasdg8-1 had a similar early flowering phenotype to its EMS-derived counterpart in *Arabidopsis* (sdg8-2). Paralleling how this mutant leads to early flowering in *Arabidopsis* (described above), the authors showed that in the Bnasdg8-1 mutant, global changes in H3K36me2/3 methylation were present and that expression of 8 of the 9 *FLC* paralogs in OSR were repressed as well as the expression of both BnaSOC1 and BnaFT were upregulated (Jiang *et al.* 2018).

Kang *et al.* (2018) transiently delivered to canola cv. Tammi protoplast plant-compatible ABE binary vectors to target precise A-to-G substitutions in the *FT* locus (TAT to CAT or CAC—antisense strand) generating a Y85H amino acid substitution in the FT protein as well as a mis-spliced RNA transcript of the phytoene desaturase gene. In using *Agrobacterium*-mediated transformation of these constructs into *Arabidopsis*, transgenic plants with late-flowering and albino phenotypes were obtained (Kang *et al.* 2018). Interestingly, the authors found (1) that base editing was only seen in the T_1 generation when the RPS5A construct was used and (2) that A-to-G conversion efficiencies were higher when T preceded a target A (Kang *et al.* 2018).

Li *et al.* (2018a) used CRISPR/Cas9 gene editing to develop a spectrum of LOF alleles early in the first exon of 5 BnaSPL3 paralogs (*BnC03.SPL3* (BnaC03g18800D), *BnA04.SPL3* (BnaA04g19840D), *BnC04.SPL3* (BnaC04g44230D), *BnA05.SPL3* (BnaA05g09840D), and *BnCnn.SPL3* (BnaCnng05200D)). The authors used hypocotyl transformation of the semi-winter OSR cv. Zhongshuang 6 using *Agrobacterium tumefaciens* with the CRISPR/Cas9 vector having a single-guide RNA (gRNA) designed to

target all 5 loci (Li *et al*. 2018a). Using PAGE analysis of annealed SPL3 amplicons, five plants exhibiting BnaSPL3 LOF mutations were identified in a screen of 72 transgenic samples (Li *et al*. 2018a). Sequencing confirmed LOF mutations in each line. Bnspl3 mutants exhibited developmental delay phenotype in the first generation. BnaSPL3, similar to its orthologs in *Arabidopsis*, are implicated in the regulation of the vegetative to floral developmental phase transition (Jung *et al*. 2012). Like *AtSPL3*, *BnaSPL3* induces the expression of *BnaAP1* (*BnaC06.AP1-1*, *BnaC06.AP1-2*, *BnaC07.AP1-1*, and *BnaC07.AP1-2*) a key MADS-box transcription factor required for this phase transition (Yamaguchi *et al*. 2009).

Once the vegetative to floral transition is initiated, a cascade of floral homeotic genes are activated (Weigel and Meyerowitz 1994). Zhang *et al*. (2018) used gene editing to develop a spectrum of mutant alleles in canola for homologs of the floral homeotic gene *APETALA2* (*AP2*), which in part underlies A function in dicot flowers. Canola has four *BnaAP2* genes, two derived from *B. rapa* and two from *B. oleracea* (Parkin *et al*. 1995; Snowdon *et al*. 2002). Zhang *et al*. (2018) used hypocotyl transformation of SOSR cv. Westar (assumed) using *Agrobacterium tumefaciens* with a CRISPR/Cas9 vector designed to make LOF mutations in the four OSR AP2 homologs (which the authors termed *BnaA-AP2a*, *BnaA-AP2b*, *BnaC-AP2a*, *BnaC-AP2b*). Of 33 transformants, 6 were confirmed to contain LOF alleles with the genotypes *BnaA-AP2a* (+1 bp), *BnaA-AP2b* (+1 and −1 bp), *BnaC-AP2a* (−4 and −5 bp), and *BnaC-AP2b* (+1 bp) with some plants showing the typical weak carpeloid or sepal carpeloid phenotypes (Zhang *et al*. 2018). For each of the four *BnaAP2* genes, these frameshift mutations led to truncated non-functional proteins. A resulting ap2 quadruple mutant exhibited the characteristic *ap2* flower phenotype (first observed in *Arabidopsis*) with carpeloid sepals, missing petals, and a reduced number of stamens, while having a normal pistil (Jofuku *et al*. 1994; Zhang *et al*. 2018).

Finally, towards developing a multilocular silique in canola, Yang *et al*. (2018) used CRISPR/Cas9 gene editing to develop a spectrum of LOF alleles by targeting the first exon and the C-terminal conserved CLV3/ESR related (CLE) domain 2 of the secreted peptide CLAVATA3 (*BnaCLV3*; *BnaA04.CLV3* (BnaA04g15710D) and *BnaC04.CLV3* (BnaC04g38990D)) or in the 5′ portion of its related receptors CLV1 (*BnaA07.CLV1* (BnaA07g32120D) and *BnaC06.CLV1* (BnaC06g36500D)) and *BnaCLV2* (*BnaA02.CLV2* (BnaA02g12070D) and *BnaC02.CLV2* (BnaC02g45200D)). The CLAVATA (CLV) pathway is functionally conserved in plants. Although *BnaC02.CLV3* (BnaC02g15230D) is present in the WOSR cv. Darmor-bzh reference sequence in Genoscope, it is not present, confirmed by Southern blotting, in the semi-winter OSR cv. J9707 (Yang *et al*. 2018). Of 860 transgenic lines generated, PAGE analysis identified 51 *Bnaclv3* mutant lines in nearly 500 samples, 47 *Bnaclv1* mutant lines in 101 samples, and 21 *Bnaclv2* mutant lines in 37 samples. Amplicons from 22 edited lines of BnaCLV3 were Sanger sequenced with 17 lines that were homozygous double mutants of Bnaclv3, 4 lines were homozygous LOF mutants in *BnA04.clv3*, and 1 line was a homozygous LOF mutant in *BnC04.clv3* (Yang *et al*. 2018). A multilocular phenotype can be recovered only when homozygous LOF mutants are obtained in both *Bnaclv*3 loci. These mutant BnaCLV3 lines produced more leaves and multilocular siliques with 33% more seeds per silique and a 25% higher seed weight than the wild-type and single mutant plants, potentially contributing to increased seed production (Yang *et al*. 2018). Although a multilocular silique phenotype was also obtained for the *Bnaclv1* and *Bnaclv2* loci, it was unstable (Yang *et al*. 2018).

Together these mutations, affecting plant height, branching, flowering time, pod number, seeds per pod, and seed weight are increasing diversity that does not currently exist in the canola gene pool and might be leveraged to increase yield in this important oilseed crop.

Plant hormones Gibberellins (GAs) are a well-characterized family of complex diterpenoid plant hormones that regulate dormancy and germination, extension growth, flowering, and fruit development in plants. In *Arabidopsis*, gibberellin-deficient dwarfs were discovered, including *Atga4* (Koornneef and van der Veen 1980). The *GA4* gene transcripts encode GA 3β-hydroxylase responsible for converting growth inactive GAs such as GA_{20} and GA_9 into their growth active forms GA_1 and GA_4 (Cowling *et al*. 1998). The *RGA* gene encodes a DELLA protein that negatively regulates GA signaling and plant growth in many plant species. By adding diversity to loci effecting gibberellin biosynthesis, both plant height (lodging resistance) and yield can be more tightly controlled.

In one of the first large-scale CRISPR/Cas9 mutagenesis papers in canola, Yang *et al*. (2017) set out to create LOF alleles in 12 genes from 4 gene families that regulate plant development, using a strategy that employed two sgRNAs for each gene to assure a high mutation rate. The gene targets included 4 paralogs of the *Arabidopsis REPRESSOR OF GA1-3* (*RGA*; Silverstone *et al*. 1997; Silverstone *et al*. 1998) gene (*BnaA9.RGA*, *BnaC9.RGA*, *BnaA6.RGA* [DS-1], and *BnaC7.RGA* [DS-3]), 3 paralogs of the *Arabidopsis FRUITFULL* (*FUL*; *BnaA9.FUL*, *BnaC2.FUL*, and *BnaC7.FUL*), and 5 paralogs of *Arabidopsis DA1* and *DA2* (DA means "large" in Chinese; *BnaA6.DA1*, and *BnaC5.DA1* as well as *BnaA2.DA2.1* (BnaA02g18880D), *BnaA2.DA2.2* (BnaA02g18890D), and *BnaC6.DA2*; Yang *et al*. 2017). Since *BnaA2.DA2.1* and *BnaA2.DA2.2* are a highly related (>98% identity) through tandem duplication, they were considered a single locus named *BnaA2.DA2* (Yang *et al*. 2017).

Of 435 T_0 transgenic lines generated, 218 were derived from constructs targeting the DELLA and TVHYNP domains within the protein BnaRGA paralogs, 115 targeted the *BnaDA* paralogs, and 102 targeted the *BnaFUL* paralogs with 53 bearing LOF mutations. Further, for all three targets, 40% of 67 lines assayed using T7 endonuclease I showed biallelic LOF edits (Yang *et al.* 2017). Of all the lines with indels in the target sites, one did not have the CRISPR/Cas9 reagents inserted randomly within the regenerated plant's genome (Yang *et al.* 2017).

Gibberellins and abscisic acid play antagonistic roles in developmental processes including germination, elongation growth, and response to drought. Focusing on the *BnaA6.RGA* [DS-1] paralog, it was determined that the expression of this gene was greatly induced by drought and abscisic acid (ABA; Wu *et al.* 2020b). A gain of function mutant of *BnaA6.RGA*, *BnaA6.rga-D* having a mutant DELLA motif, displayed enhanced drought tolerance, with its stomatal closure being hypersensitive to ABA treatment whereas the quadruple LOF *Bnarga* mutant was less drought tolerant and was also less sensitive to ABA treatment (Wu *et al.* 2020b). The *Bnarga* single mutants failed to show a difference in drought tolerance compared to the WT control cv. Westar inferring that these genes are functionally redundant in this process (Wu *et al.* 2020b).

Targeting the conserved TVHYNP domain of 2 *BnaRGA* paralogs (BnaA09g18700D and BnaC09g52270D) in the semi-winter OSR cv. Zhongshuang 6, Cheng *et al.* (2021—as described in the Herbicide section above) employed a CBE to introduce C-to-T or G-to-A transition mutations in the editing window. Of 63 transgenic plants targeting these loci, 2 plants had a CCG to CTG codon substitution encoding a P94L amino acid substitution in the BnaA09g18700D locus. Additionally, due to G-to-T and T-to-C base conversion, 16 plants had a CCG to TTG codon substitution encoding a P94L amino acid substitution in the BnaC09g52270D locus since the gRNA perfectly matched the sequence of this locus. As expected, semi-dwarf mutants generated in this approach phenocopy the OSR ds-3 mutant (Zhao *et al.* 2017).

Cheng *et al.* (2021—as described above) also used a CBE to target C-to-T or G-to-A transition mutations in the conserved motif (GWPPV) of *BnaIAA7* paralogs (BnaC01g43640D, BnaA03g36950D, BnaA05g16680D, and BnaC05g29300D) in the semi-winter OSR cv. Zhongshuang 6. Of 32 transgenic plants targeting these loci, 10 plants had transition mutations in the editing window for each of the BnaC01g43640D, BnaA03g36950D, and BnaA05g16680D loci, with 7 plants having edits in the BnaC05g29300D locus. Of the mutations that were shown for the BnaC05g29300D locus, all 4 plants had a CCT to TTT codon substitution encoding a P94L amino acid substitution (Cheng *et al.* 2021). Similar to the phenotype observed when the homolog of this gene is mutated in *Arabidopsis*, seedlings exhibit a dwarf phenotype with crinkled leaves and mature plants being extremely small and very late flowering.

Lin *et al.* (2018) also targeted LOF mutations in the *GA4.a* gene in canola protoplasts. The authors showed indels in a protoplast population that was treated with a plasmid expressing the CRISPR/Cas9 nuclease and the relevant gRNA, but did not regenerate plants from this population.

Unlike most of the transgenic work that preceded it, Tang *et al.* (2018) used *Agrobacterium*-mediated transformation of hypocotyls and visual selection of GFP positive plants in combination with antibiotic selection to identify LOF lines generated with CRISPR/Cas9 reagents. For their experiments with canola cv. Westar, more than 50% of the stable transformants had LOF mutations in the target genes (Tang *et al.* 2018). This visual selection system enabled LOF mutants to be obtained in all 4 canola *AUXIN RESPONSE FACTOR2* (*BnaA6.ARF2*, *BnaA9.ARF2*, *BnaC3.ARF2*, and *BnaC9.ARF2*) loci, a repressor in auxin signaling (Schruff *et al.* 2006; Tang *et al.* 2018). Further, 5 GFP-positive plants were observed and confirmed to be PCR positive using Cas9-specific primers, with Sanger sequencing of the target regions used to confirm 1 to 3 LOF alleles per target locus (Tang *et al.* 2018). Of these transgenic lines, 3 were quadruple mutants, 1 was a triple mutant (*BnaA9.ARF2*, *BnaC9.ARF2*, and *BnaC3.ARF2*), and the last line was double mutant (*BnaC9.ARF2* and *BnaC3.ARF2*; Tang *et al.* 2018). Compared with the cv. Westar parent, the phenotypes of these Bnaarf2 mutants included shorter primary roots, a significantly increased seed size and 1000-seed weight, suggesting that, like its role in *Arabidopsis*, BnARF2 is a positive regulator of root elongation and a negative regulator of seed enlargement (Schruff *et al.* 2006; Tang *et al.* 2018).

Architecture Strigolactone, an emerging plant hormone that controls root growth, shoot branching, and interactions with symbiotic fungi and parasitic weeds, is a cleavage product of β-carotene (Xie *et al.* 2010). Stanic *et al.* (2020) generated CRISPR/Cas9-mediated LOF mutations in genes encoding the strigolactone receptor D14 in each of the A (LOC106435377) and C (LOC106431289) genomes following hypocotyl transformation of canola cv. Westar using *Agrobacterium*. Two primary transformants with a dwarf phenotype were obtained bearing biallelic LOF mutations in each of the 2 targeted loci (Stanic *et al.* 2020). Mean plant height was reduced 34% resulting from a reduced internode length. These mutant plants also branched prolifically (200% more than the cv. Westar parental control) and had 37% more flowers leading to slight and non-significant increases of 12% and 10% in pod weight and seed yield, respectively (Stanic *et al.* 2020).

Zheng *et al.* (2020) also made LOF alleles in homologous gene targets that control lateral branching. A CRISPR/Cas9 approach was used to generate LOF mutations in genes

encoding the 2 canola *BnaMAX1* homologs (BnaA03g22900D and BnaC03g26960D). MAX1 is an enzyme involved in strigolactone biosynthesis encoding a cytochrome P450 monooxygenase (CYP711A1) that converts carlactone to carlactonoic acid (Booker *et al.* 2005). The authors accomplished this by *Agrobacterium*-mediated transformation of the SOSR cv. 862 hypocotyls. Of the 4735 calluses obtained, 141 hygromycin-resistant positive transgenic plants were selected with 29 of these bearing LOF alleles in the target loci (Zheng *et al.* 2020). Unexpectedly, 3 T_0 lines had large deletions (85–165 bp) causing them to lose the entire sgRNA target sites (Zheng *et al.* 2020). Of the 29 plants with LOF alleles, 4 lines bearing a diverse set of LOF alleles were taken to the next generation with some of these progeny being null segregants for the transgene. Plants with homozygous LOF alleles in both *BnaMAX1* loci were 33% shorter, with a 2-fold increased branching, having 33% more siliques, all of which contributed to a 25% increased yield per plant relative to the parental control (Zheng *et al.* 2020).

In summary, the work of Stanic *et al.* (2020) and Zheng *et al.* (2020) produced diversity in plant architecture that resulted in better mechanical harvest, reduced lodging, and increased yield.

Self-incompatibility Genetic systems for self-incompatibility (SI) are elaborate mechanisms that have evolved independently multiple times to promote outcrossing and maintain genetic diversity in many flowering plants. Each SI system prevents hydration, germination, and growth of self-incompatible pollen tubes. In the *Brassicaceae*, the SI system has two components encoded at the complex and polymorphic S-locus, (i) the transmembrane receptor S locus receptor kinase (SRK) expressed predominantly in the stigma and (ii) its ligand the S locus cysteine-rich (SCR) protein expressed in the tapetum that becomes part of the pollen coat (Nasrallah 1997; Schopfer *et al.* 1999; Suzuki *et al.* 1999). Commercially, SI systems can be used for developing hybrids. In *Brassica napus*, the M-locus protein kinase (MLPK) BnaMLPKs has four paralogs, *BnaA3.MLPK*, *BnaC3.MLPK*, *BnaA4.MLPK*, and *BnaC4.MLPK*, which control SI (Chen *et al.* 2019). *Agrobacterium*-mediated transformation of CRISPR/Cas9 reagents was used to make loss of function alleles in each target gene, using the method of Yang *et al.* (2017), with two independent gRNAs per locus. In assessing 48 transgenic lines, 6 per gRNA per locus, 44 plants were determined to bear LOF alleles in one of the four target loci (Chen *et al.* 2019). Six of these plants were crossed to obtain two Bnamlpk quadruple mutants. Compared with the SI line S-70, the SI response was completely blocked in these quadruple mutants as evidenced by pollen germination, pollen tube elongation, and excellent seed set (Chen *et al.* 2019). By contrast, when pollen from the quadruple mutants was used to pollinate SI line S-70, no pollen germination or seed set was observed confirming that BnaMLPKs are not the pollen SI determinants. Expression of six genes known to be associated with SI was assessed by qRT-PCR with three being differentially expressed in the Bnamlpk quadruple mutant compared to the SI line (Chen *et al.* 2019). Before pollination, the expression of the SRK and arm repeat containing 1 (ARC1), an E3 ubiquitin ligase, were suppressed in the Bnamlpk quadruple mutant disrupting SI signal transduction to enable pollen tube growth and elongation. Both MLPK and ARM1 are positive regulators that are known to interact with SRK in the SI pathway (Shi *et al.* 2016). By comparison, the self-compatible factor GLO1 was slightly induced in the Bnamlpk quadruple mutant (Chen *et al.* 2019). With or without pollination, 3 genes including thioredoxin h-like proteins THL1 and THL2 as well as Exo70A1 were not differentially expressed between the mutant and SI line S-70 (Chen *et al.* 2019). This work implicates the four BnaMLPK loci in the SI response in *Brassica napus* extending what was known from studying this response in *Arabidopsis* and *Brassica oleracea*.

Male sterility Hybrid systems enable increased plant vigor, disease resistance, and yield. The male sterility system 5 (MS5) gene underlies a three-line hybrid system in *Brassica napus* with the restorer allele being $MS5^a$, the male-sterile allele being $MS5^b$ and the maintainer allele being $MS5^c$ (Lu *et al.* 2012). This *Brassica*-specific nuclear localized gene encodes a protein containing conserved N-terminal coiled coil (CC) and C-terminal DUF626 domains (Xin *et al.* 2016). When compared with $MS5^a$, an 8-kb Mutator-like transposable element (MULE) occurs in the second intron of $MS5^b$ (Xin *et al.* 2016). The $MS5^a$ and $MS5^c$ alleles are significantly divergent in both expression levels and amino acid sequence, displaying 18 amino acid differences over their more than 300 amino acid length (Xin et al., 2016). Towards understanding this triallelic sterility system, focusing on obtaining LOF alleles in the fertile lines cv. Westar ($MS5^c/MS5^c$) and Y127 ($MS5^a/MS5^a$), Xin et al. (2020) used *Agrobacterium*-mediated transformation CRISPR/Cas9 vectors with two gRNAs targeting the CC domain of this gene target, homozygous or biallelic ms5 mutants from 5 of 8 T_0Y127 plants and 2 of 20 T_0 cv. Westar plants were male sterile (Xin et al. 2020). Compared with the WT, chromosomes were gathered as a compact mass in these engineered mutants at approximately the tetrad stage demonstrating that both $MS5^a$ and $MS5^c$ are necessary for male fertility by participating in the regulation of early meiosis in *B. napus* (Xin et al. 2020).

In a different, and more typical, two-line hybrid system, production of hybrid seed requires two lines, one line having a male sterile phenotype usually caused by a maternally inherited cytotoxic cytoplasmic male sterility (CMS) trait encoded in the mitochondrial genome, and the second line bearing a nuclear fertility restoring (Rf) gene (Iwabuchi *et al.* 1999). The mitochondrial gene orf125 is a CMS-

associated gene in Kosena-type CMS (Iwabuchi *et al.* 1999). Using the method of Kohno-Murase *et al.* (1994), Ti plasmids expressing TALENs targeted to the mitochondria (termed mitoTALENs) were integrated into the nucleus of Kosena-type CMS *Brassica napus* L. (SW18) with transformants being regenerated under kanamycin selection (Kazama *et al.* 2019). Line SW18 is a cybrid that was originally created by asymmetric cell fusion between canola cv. Westar and a CMS radish (*Raphanus sativus* L. 'Kosena') and extensively backcrossed to cv. Westar to recover that parental phenotype (Sakai and Imamura 1992). Five transgenic lines were obtained, with PCR amplification confirming that 3 lines lacked the mitochondrial locus orf125 (Kazama *et al.* 2019). The fertility of all 3 lines lacking orf125 was restored confirming that this locus is responsible for the CMS phenotype in the Kosena-type CMS system (Kazama *et al.* 2019). This peer-reviewed publication demonstrates that gene editing can also be performed in the mitochondrial genome of *Brassica napus*.

Nutrition Canola is extremely sensitive to boron deficiency (Marschner 2012), preventing seed set even when flowering appears normal (Xu *et al.* 2002). Feng *et al.* (2020) used *Agrobacterium*-mediated hypocotyl transformation of canola cv. Westar 10 using a CRISPR/Cas9 vector designed to make LOF mutations in the transcription factor *BnaA9.WRKY47* (BnaA09g00350D). From 19 independent mutant lines, null segregants for 3 BnaA9.WRKY47 homozygous mutants were studied further. These lines had higher sensitivity to low boron content than WT plants demonstrating that *BnaA9.WRKY47* contributes to the adaptation of canola to low boron stress (Feng *et al.* 2020). In part, this is likely since BnaA9.WRKY47 directly activates the expression of a boric acid channel NIP (nodulin 26-like intrinsic protein) BnaA3.NIP5;1 and BnaC3.NIP5;1 in roots under boron deficiency (Feng *et al.* 2020). This is reinforced by the fact that BnaA3.NIP5;1 contains a conserved W box cis element in this downstream gene's promoter and that it is bound by BnaA9.WRKY47 in an electrophoretic mobility shift assay (Feng *et al.* 2020).

Disease resistance Disease resistance is a co-evolutional arms race between plant and pathogens. Even when suitable fungicides are available, a suitable application window may not be available and mutations in these rapidly evolving organisms may make them less effective. What's more, consumers are gravitating to organically produced foods and many governments are mandating the reduction of fungicides as part of "green deals" thus elevating a need for genetic disease resistance traits. Providing that yield and quality are not impacted, improving genetic disease resistance is therefore the preferred path.

Pathogens that can infect *Brassica* crops and cause production losses include viruses, bacteria, fungi, and oomycetes. Yield, seed quality, and crop development are significantly impacted by pathogens of canola, which include the chytrid *Plasmodiophora brassicae* (club root); fungi including *Leptosphaeria maculans* (Blackleg or stem canker); the multinucleate filamentous pathogen *Sclerotinia sclerotiorum* (*Sclerotinia* stem rot or white mold), *Alternaria brassicae* (*Alternaria* Blight), *Albugo candida* (White Rust), *Pseudocercosporella capsellae* (White Leaf Spot), and *Verticillium longisporum* (*Verticillium* stem striping); the oomycete *Hyaloperonospora parasitica* (Downy Mildew); bacteria including *Pseudomonas syringae* (Blackrot); and viruses including the aphid transmitted Turnip Mosaic Virus (Murray and Brennan 2012).

Sclerotinia stem rot is a key disease in canola that can lead to poor oil quality and cause grain yield losses ranging from 10 to 80% (Purdy 1979). Infection of canola occurs when ascospores colonize senescent flower petals that have dropped onto leaves or petioles adjacent to the stem, followed by the development of necrotic lesions on the stem causing it to weaken or break and the plants to lodge and wilt. In canola, *BnWRKY11* and *BnWRKY70* genes are differentially expressed after inoculation with *Sclerotinia sclerotiorum* (Lib.) de Bary (Wu *et al.* 2016); in *Arabidopsis*, these single-copy loci are implicated in jasmonic acid (JA)– and salicylic acid (SA)–induced resistance to pathogens (Journot-Catalino *et al.* 2006; Hu *et al.* 2012). In canola, there are 6 paralogous loci that encode each of *BnaWRKY11* (*BnaA.WRKY11.a* (BnaA03g51590D), *BnaC.WRKY11.a* (BnaC07g43320D), *BnaA.WRKY11.b* (BnaA01g34790D), *BnaC.WRKY11.b* (BnaC01g06900D), *BnaA.WRKY11.c* (BnaA08g12420D), *BnaC.WRKY11.c* (BnaC03g67520D)) and *BnaWRKY70* (*BnaA.WRKY70.a* (BnaA07g165850D), *BnaC.WRKY70.a* (BnaC06g15910D), *BnaA.WRKY70.b* (BnaA09g35840D), *BnaC.WRKY70.b* (BnaC08g27340D), *BnaA.WRKY70.c* (BnaA04g02560D), *BnaC.WRKY70.c* (BnaCnng52600D)). Sun *et al.* (2018) used a CRISPR-Cas9 approach to inactivate the 2 *BnaWRKY11* (*BnaA.WRKY11.a* (BnaA03g51590D) and *BnaC.WRKY11.a* (BnaC07g43320D)) and 4 *BnWRKY70* paralogs (*BnaA.WRKY70.a* (BnaA07g165850D), *BnaC.WRKY70.a* (BnaC06g15910D), *BnaA.WRKY70.b* (BnaA09g35840D), and *BnaC.WRKY70.b* (BnaC08g27340D)). These selected loci had the highest level of expression prior to inoculation, and following inoculation with *Sclerotinia* were most differentially expressed with the two *BnaWRKY11* paralogs showing the greatest increase in expression, and the 4 *BnaWRKY70* paralogs showing the greatest decrease in expression (Sun *et al.* 2018). Arising from transformation of hypocotyl segments of SOSR cv. J9712 using *Agrobacterium*, 30 PCR positive T_0 transgenic plants with the *NPTII* selectable marker were obtained, 22 for the BnaWRKY11 target and 8 for the BnWRKY70 target. Of those, 12 lines had mutant alleles in *BnaWRKY11* (2 different ones in *BnaA.WRKY11.a* with 23 in

BnaC.WRKY11.a) and 4 lines had mutant alleles *BnaWRKY70* (4 different ones in *BnaA.WRKY70.a* with 1 in *BnaA.WRKY70.b*). Given the gRNAs that were used, a deletion of 302 bp between the 2 gRNAs was observed in 1 of the edited *BnaC.WRKY11.a* lines (Sun *et al*. 2018). All edits in both *BnaWRKY11* and *BnaWRKY70* were indels with no substitutions observed (Sun *et al*. 2018). Editing chimeras were observed in two lines, 1 for each of *BnaWRKY11* and *BnaWRKY70* (Sun *et al*. 2018). Finally, for at least the *BnaWRKY11* target, mutagenesis was not observed for some gRNAs in the T_0 generation, but in at least 2 lines it was detected in the T_1 generation (Sun *et al*. 2018).

The vast majority of *Bnawrky70* mutants showed editing in three paralogs of *BnaWRKY70* in examined T_1 plants. Based on the results of a detached leaf assay 48 h post-infection, homozygous triple mutants in *Bnawrky70* (*BnaA.wrky70.a*, *BnaA.wrky70.b*, and *BnaC.wrky70.b*) showed enhanced resistance to *Sclerotinia*, while homozygous double mutants in *BnaWRKY11* (*BnaA.wrky11.a* and *BnaC.wrky11.a*) failed to show a significant difference in resistance when compared to the parental check (Sun *et al*. 2018). By comparison, also based on the results of a detached leaf assay, transgenic plants that overexpressed BnaWRKY70 were more sensitive to this pathogen compared to the parental check (Sun *et al*. 2018). Taken together, these results show that LOF mutations in *BnaWRKY70* might be used to achieve tolerance to *Sclerotinia* since this transcription factor functions as a negative regulator of *Sclerotinia* resistance in canola (Sun *et al*. 2018).

The hemibiotrophic fungal pathogen *Verticillium longisporum* is a soil-borne pathogen that infects the *Brassicaceae* causing stem striping (Depotter *et al*. 2016). Plants are infected when microsclerotia recognize root exudate from the host plant, germinate, and follow the nutrient gradient to reach the root. The fungus gains access to the plant by directly penetrating its epidermis or by taking advantage of wounds before its hyphae spread intra and intercellularly, ultimately producing conidia that are transported by the vascular system (Depotter *et al*. 2016). Eventually, this fungus begins its necrotrophic life phase in which it feeds on senescing leaves and stems.

In canola, transcriptome analysis showed a cohort of activated/upregulated genes 5 to 15 d following *Verticillium* (*Vl43*) infection (Pröbsting *et al*. 2020). Initially, T-DNA insertion LOF mutants for one of these genes, *AtCRT1a* (At1g56340), was identified in *Arabidopsis*. In this mutant, the ethylene signaling pathway is activated and is less susceptible to *Vl43* infection (Pröbsting *et al*. 2020). Next, the mutagenesis approach termed Targeting Induced Local Lesions IN Genomes (TILLING) approach identified 10 mutations in the *BnaCRT1a* paralog *BnaA09.CRT1a* (BnaA09g15400D), then using a CRISPR/Cas9 approach LOF alleles were generated in 2 *BnCRT1a* paralogs (paralogs *BnaA09.CRTa* (BnaA09g15970D) and *BnaC09.CRT1a* (BnaC09g16150D))—with the gRNA designed to discriminate against *BnaA09.CRT1a* paralogs (BnaA09g15970D and BnaC01g43040D; Pröbsting *et al*. 2020). The authors accomplished this by hypocotyl transformation of cv. Express using *Agrobacterium tumefaciens* (Pröbsting *et al*. 2020). Of 20 transformants, 4 lines had confirmed LOF mutations in *BnaA09.CRTa* (5 LOF alleles) and *BnaC09.CRT1a* (12 LOF alleles). Three lines were biallelic in one *BnaCRT1a* paralog and the fourth was biallelic in both paralogs. In the TILLING approach, one mutant line identified was hypersensitive to *Vl43* infection with two others being more resistant. Without a time-consuming backcross to clear the mutant line of mutations not associated with this phenotype, this result was inconclusive. In the CRISPR/Cas9 approach, the *Bnacrta* mutant plants confirmed that *BnaA09.CRTa* has a dominant role in a compatible interaction with this fungus and, therefore, when this locus bears homozygous LOF alleles, susceptibility to *Vl43* is decreased (Pröbsting *et al*. 2020). As is the case in the *Atcrta* LOF mutant, ethylene signaling was also activated in the *Bna A09.crt1* mutant. Together, in a crop with limited natural resistance to *Verticillium*, these results show a promising path towards achieving resistance to this devastating pathogen.

Understanding host-pathogen interaction by pathogen editing Globally, blackleg (*Leptosphaeria maculans*) is the most important disease affecting canola causing losses exceeding $900 M annually (Fitt *et al*. 2008). There are numerous reports of resistance genes (mainly QTLs), but very few have been cloned and characterized for this pathogen. In specific gene for gene interactions of a pathogen with its host, there is a specific interaction of an avirulence (Avr) gene (e.g., *UmAvr7*) with its corresponding R gene (e.g., *Rlm7*). In this example, the blackleg pathogen bearing *UmAvr7* is virulent on hosts that do not have the resistance gene *Rlm7*. Not only can disease resistance be studied from the host (crop) side, but also by editing the pathogen.

In a disease survey conducted in western Canada, a total of 180 Avr gene races were identified from 964 isolates, with three major races observed: AvrLm-2-45-6-7, AvrLm2-4-5-6-7-S, and AvrLm-1-4-5-6-7-11-(S). The UMAvr7 isolate carried AvrLm1-2-3-4-9-11-LepR1LepR2-S-AvrLm5-6-7 and is PCR positive for AvrLm5, AvrLm6, and AvrLm4-7 (Zou *et al*. 2020). Previously, Parlange *et al*. (2009) determined that when codon358 in AvrLm4-7 has a transversion of "C" to "G", this pathogen is recognized by Rlm7 and Rlm4/7 in canola.

Zou *et al*. (2020) used *Agrobacterium* transformation of *L. maculans* isolate (DS103) (named UMAvr7) to make LOF alleles of the avirulence gene target *AvrLm4-7* as previously described by Gardiner and Howlett (2004). The resulting mutant isolates were tested on different canola genotypes with 6 plants per genotype inoculated as described by Zhang *et al*. (2016). Among the mutant isolates, Mu3,

hereafter termed as *umavr7*, when inoculated onto canola line 01-232-1 (Rlm7), developed large disease lesions similar to those observed in the susceptible cv. Westar, indicating a shift from avirulence to virulence. Further, by assessing H_2O_2 accumulation in cotyledons of resistant genotype 01-23-2-1, UMAvr7 caused limited accumulation around the wound site compared to mutant isolate umavr7 (Zou *et al.* 2020).

Recently, Jiquel *et al.* (2021), using a CRISPR-Cas9 editing approach in the blackleg pathogen and starting with a short list of 5 late effector candidates, were able to analyze the interaction of a blackleg (*L. maculans*) late effector (avirulence) *LmSTEE98* gene involved in the biotrophy to necrotrophy transition with *RlmSTEE98*, a cognate resistance gene in canola that responds to this effector. Initially, narrowing down from a set of more than 200 canola candidate genotypes, it was determined that cv. Yudal elicited a hypersensitive resistance response to this effector. To validate this interaction, using the method described by Idnurm *et al.* (2017), CRISPR-Cas9 was used to make a panel of mutant alleles in *LmSTEE98* in *L. maculans*. Four independent mutants with four different indels (+1, −2, −9, −17 bp) were obtained and these putative LOF alleles were tested in a cotyledon stage model system assay to confirm that out-of-frame indels abolished this interaction, whereas the in-frame (−9 bp) deletion remained avirulent (Jiquel *et al.* 2021). Further, in a stem canker assay in *B. napus* cv. Yudal, isolates bearing the three out-of-frame *Lmstee98* mutants elicited a stem necrosis resistance response, whereas the one bearing the in-frame (−9 bp) deletion remained avirulent. All four isolates remained virulent in *B. napus* cv. Darmor-bzh, the susceptible check. With these tools in hand, the authors used a mapping approach using progeny from a dihaploid population developed from a cross of Darmor-bzh x Yudal to narrow the hypersensitive resistance response to a 350-kb interval on chromosome A09 bearing the cognate resistance gene (Jiquel *et al.* 2021). This region contains 70 genes, based on the reference genome of the susceptible check cv. Darmor-bzh (Jiquel *et al.* 2021).

Li *et al.* (2018b) used a CRISPR/Cas9 approach aimed at achieving LOF alleles in the oxalate biosynthesis gene *Ssoah1* GenBank accession number XM_001590428 in the necrotrophic pathogen *Sclerotinia sclerotiorum* (Lib.) de Bary (UF isolate) by delivering a plasmid encoding CRISPR/Cas9, gRNA expression cassette, and a hygromycin selectable marker. After three rounds of selection on potato dextrose agar (PDA) supplemented with the pH indicator bromophenol blue, the media surrounding strains that did not accumulate the chemical effector oxalic acid were also resistant to the selection agent hygromycin (Li *et al.* 2018b). Of a total of 92 hygromycin-resistant transformants, 38 (41%) failed to acidify the growth media (Li *et al.* 2018b). In the parental mutant, rather than having small indels at the target site, large insertions, likely to be tandem insertion of the vector, disrupted the target gene (Li *et al.* 2018b). In meiosis to produce ascospores, tandem insertions undergo intrachromosomal recombination to reduce the tandem insertion to a single copy (Li *et al.* 2018b). Like a previously described mutant Δss-oah1 in the 1980 strain (Liang *et al.* 2015), mutants lacking OAH1 function have enhanced production of compound appressorium, show decreased pigmentation on the surface of sclerotia, and in culture display a diffuse pattern of sclerotium development (Li *et al.* 2018b)

Seed-related gene editing targets-Yellow-seeded canola Zhai *et al.* (2020) using hypocotyl transformation of the semi-winter OSR cv. J9707 with *Agrobacterium tumefaciens* used CRISPR/Cas9 with guide RNA designed to target the basic helix-loop-helix (bHLH) transcription factors *TRANSPARENT TESTA8 BnaA09.TT8* (BnaA09g22810D) and *BnaC09.TT8b* (BnaC09g24870D) to make a yellow-seeded *B. napus*. A tandemly duplicated gene *BnaC09.TT8a* (BnaC09g24860D) lacked a critical conserved domain and did not appear to be transcribed. It was presumed to be a pseudogene and was not targeted. Transformation resulted in 333 PCR positive events (Zhai *et al.* 2020) producing a total of 48 targeted mutants identified by sequencing of the PCR products from the target sites (Zhai *et al.* 2020). Five plants, all determined to be homozygous double mutants, showed a visible LOF (yellow-seeded) phenotype, whereas the single homozygous mutants had the parental phenotype confirming that these two genes function redundantly (Zhai *et al.* 2020). Homozygous mutations at the target sites within *BnaTT8*, mainly due to frame shifts, were determined to cause non-functional proteins (Zhai *et al.* 2020). By blocking the proanthocyanidin-specific deposition in the inner layer of the seed coat, the desired phenotype was obtained in lines bearing LOF mutations in both *BnaTT8* functional copies with this double mutant having elevated seed oil, likely resulting from its thinner seed coat, and protein content in addition to an altered fatty acid (FA) composition (Zhai *et al.* 2020). In *B. rapa* and *B. juncea*, natural LOF mutants in these genes lead to a yellow seeded trait (Li *et al.* 2012a, 2012b; Padmaja *et al.* 2014).

Xie *et al.* (2020) used hypocotyl transformation of cv. J9712 with *Agrobacterium tumefaciens*, using CRISPR/Cas9 with gRNAs designed to target LOF alleles in the R2 and R3 domain of the Myb123 transcription factors *BnaA08.TT2* (BnaA08g29930D) and *BnaC08.TT2* (BnaC08g07960D) towards achieving a yellow-seeded canola. Following selection, 81 transformants had PCR products from the target sites sequenced identifying a total of 4 plants with targeted mutant alleles, three alleles for each of *BnaA08.TT2* and *BnaC08.TT2* (Xie *et al.* 2020). In the T_0 generation, 2 plants had a mutant allele in either *BnaA08.TT2* or *BnaC08.TT2* and the other two

with biallelic mutations in either *BnaA08.TT2* or *BnaC08.TT2* and hemizygous mutations in the other allele; no homozygous mutant lines were obtained (Xie *et al*. 2020). As was the case for *BnaTT8*, homozygous *BnaTT2* double mutants were yellow-seeded due to significantly reduced flavonoid content, especially epicatechin and isorhamnetin, increased oil content and improved fatty acid composition with more polyunsaturated fatty acids (Xie *et al*. 2020). Yellow seeds with homozygous *BnaTT2* double LOF mutants also had reduced lignin and synaptic acid (Xie *et al*. 2020).

Reduced phytate Phytic acid is the principle stored form of phosphorus in plants but is considered anti-nutritive for monogastric animals including humans because it chelates minerals that impede their absorption. This is due to phytases in these animals being unable to hydrolyze it to free inorganic phosphate and myo-inositol. Further, phytic acid, which accounts for 2–5% of canola grain (Sashidhar *et al*. 2020), if left undigested,, passes through the gastrointestinal tract causing environmental problems including eutrophication that threatens aquatic life. Globally, more than 400 dead zones caused by eutrophication exist. Inositol tetrakisphosphate kinase (ITPK) catalyzes the penultimate step in the synthesis of phytic acid in plants (Raboy 2009). Sashidhar *et al*. (2020) used a CRISPR-Cas9 approach to inactivate the 15 paralogs in the canola *ITPK* gene families: *BnaITPK1* (*BnaA03.ITPK1* (BnaA03g06170D), *BnaC03.ITPK1* (BnaC03g07940D), *BnaA10.ITPK1* (BnaA10g17710D), and *BnaC09.ITPK1* (BnaC09g41080D)), *BnaITPK2* (*BnaA01.ITPK2* (BnaA01g03220D), *BnaC01.ITPK2* (BnaC01g04480D), *BnaA03.ITPK2* (BnaA03g50630D), *BnaC03.ITPK2* (BnaC03g66400D), *BnaAnn.ITPK2* (BnaAnng34680D), and *BnaC07.ITPK2* (BnaC07g44490D)), *BnaITPK3* (*BnaA03.ITPK3* (BnaA03g38640D) and *BnaCnn.ITPK3* (BnaCnng47190D)), and *BnITPK4* (*BnaA05.ITPK4* (BnaA05g03660D), *BnaC04.ITPK4a* (BnaC04g03240D), and *BnaC04.ITPK4b* (BnaC04g03250D)). After *Agrobacterium* transformation of 321 hypocotyl segments of the SOSR cv. Haydn and selecting for the transgene, 23 shoots from 10 independent transgenic events, all PCR positive for Cas9 as part of the transgene, were regenerated to whole plants (Sashidhar *et al*. 2020). Each independent transgenic line had LOF alleles in up to 4 gene families (Sashidhar *et al*. 2020). Further analysis of 3 lines using Sanger sequencing of PCR amplicons revealed 3 to 8 mutant alleles per locus except *BnaA03.ITPK1* and BnaC03.ITPK1, for which no edited alleles were observed (Sashidhar *et al*. 2020). At the T_4 generation, 2 triple LOF locus mutants (in *BnaA10.ITPK1*, *BnaC09.ITPK1*, and *BnaC04.ITPK4* b) exhibited low phytic acid (with a 30% decrease) and a concomitant increase (approximate doubling) of free phosphorus (Sashidhar *et al*. 2020). This work describes an important advance towards improving meal quality in canola.

Oil seed content Using a combination of chemical mutagenesis and transformation of hypocotyl segments of WOSR cv. RS306 using *Agrobacterium tumefaciens* containing the CRISPR/Cas9 reagents, Karunarathna *et al*. (2020) aimed to increase seed oil content (and thus oil per acre) by producing LOF alleles in the *SEED FATTY ACID REDUCER* genes *BnSFAR4* (*BnaA06.SFAR4a*, *BnaA06.SFAR4b*, *BnaC03.SFAR4 a*, and *BnaCxx.SFAR4.b*) and BnSFAR5 (*BnaA03.SFAR5* and *BnaC07.SFAR5*)—from the GDSL lipases. To date, about 1100 GDSL lipases/esterases have been found in plants with this family consisting of 105 members in the model plant *Arabidopsis* (Lai et al. 2017). Eight hundred fifty-seven, 442, and 754 excised hypocotyls were transformed to achieve LOF alleles in *BnaSFAR1*, *BnaSFAR4*, and *BnaSFAR5* yielding, 2, 5, and 2 transgenic plants, respectively (Karunarathna *et al*. 2020). For *BnaSFAR4*, 13 different mutant alleles with single nucleotide insertions and deletions were made with each mutation producing a premature stop codon leading to a truncated protein (Karunarathna *et al*. 2020). Mutant segregation was Mendelian. The authors attempted to segregate away the transgene and were successful for *BnaSFAR4*, but not for *BnaSFAR5*, which was assumed to be due to a complex insertion pattern (Karunarathna *et al*. 2020). Interestingly, the *Bnasfar4* mutant lines showed significantly larger oil bodies (Karunarathna *et al*. 2020). Seed oil content increased late in the seed maturation phase by making LOF mutations in *Bnasfar4* (9.7–14.5%) and *Bnasfar5* (<10%) genes without adversely affecting seed germination or vigor (Karunarathna *et al*. 2020).

Targeting the largest number of homologous loci to date, Zhang *et al*. (2019) used hypocotyl transformation of the semi-winter OSR cv. J2016 using *Agrobacterium tumefaciens* with the CRISPR/Cas9 vector having 3 guide RNAs designed to target conserved regions in the 7 homologous *BnaLPAT2* genes (2 *BnaA07.LPAT2* loci, *BnaC07.LPAT2*, *BnaA09.LPAT2*, *BnaC08.LPAT2*, *BnaA04.LPAT2*, *BnaC04.LPAT2*) and a single-guide RNA designed to target a conserved region in the 4 homologous *BnaLPAT5* genes (*BnaA05.LPAT5-*, *BnaC05.LPAT5-*, *BnaC01.LPAT5-*, and *BnaUnk.LPAT5-*). Respectively, the *BnaLPAT2* and *BnaLPAT5* families were 60.0% and almost 80% identical suggesting that their functions in oil biosynthesis have diverged (Zhang *et al*. 2019). Of 247 regenerated plantlets, 227 were PCR positive for the transgene, with target region sequencing revealing 100 bearing LOF alleles in each of the 7 *BnaLPAT2* and 4 *BnaLPAT5* gene targets. As would be expected, when multiple gRNAs were combined on a single construct, deletions of the regions between the target sites were obtained (Zhang *et al*. 2019). No off-target editing of 14 potential off-target sites with 1, 2, or 3 mismatches was detected (Zhang *et al*. 2019). Phenotypically, seeds with mutant *Bnalpat2* and *Bnalpat5* genes had a decreased oil content

(24–39%), displayed a wrinkled phenotype, and had fewer larger oil bodies perhaps as a result of smaller oil bodies coalescing; protein bodies were affected, and starch accumulated in mature seeds (Zhang *et al*. 2019). Specifically, when the BnaLPAT2 targets were disrupted, on average C18:0 and C20:0 increased 60% and 90%, respectively, whereas C18:1, C18:2, and C18:3 decreased on average 8%, 8%, and 21%, respectively.

High oleic oil Compared with other vegetable oils, canola oil is high in unsaturated fatty acids comprised of monounsaturated oleic acid and an optimal 2:1 ratio of the polyunsaturated linoleic and linolenic acid (Hu *et al*. 2006). Vegetable oils with higher oleic acid (C18:1) contents are desirable because of their higher thermal stability and improved shelf life. This can be important for applications in both food and industrial applications. In plants, the stearoyl-acyl carrier protein desaturase encoded by the *FATTY ACID DESATURASE 2* (*FAD2*) genes catalyzes desaturation of stearic acid (C18:0) to oleic acid (C18:1). The *B. napus* genome contains four *FAD2* orthologues with two genes originating from *B. rapa* (*BnaA05.FAD2a* (BnaA05g26900D) and *BnaA01.FAD2b* (BnaA01g09250D)) and the other two from *B. oleracea* (*BnaC05.FAD2a* (BnaC05g40970D) and *BnaC01.FAD2b*; Yang *et al*. 2012). Three copies (*BnaA05.FAD2a*, *BnaC05.FAD2a*, and *Bna05C.FAD2b*) appear intact and likely to be functionally redundant, in contrast to *BnaA01.FAD2b*, which appears to be a non-functional pseudogene resulting from a single bp deletion at nucleotide 164 that leads to a premature stop codon at position 411 (Yang *et al*. 2012; Lee *et al*. 2013; Well *et al*. 2014; Bai *et al*. 2019; Huang *et al*. 2020).

Okuzaki *et al*. (2018) used hypocotyl transformation of cv. Westar using *Agrobacterium tumefaciens* with the CRISPR/Cas9 vector designed to target *BnaFAD2a* in both the A and C genomes. Out of 20 T_0 transgenic plants obtained, three had LOF alleles within the gene target with only two transmitting the LOF allele to their progeny (Okuzaki *et al*. 2018). One of these plants was backcrossed to the parent cv. Westar to generate 3 null segregants for the transgene (containing the selectable marker and CRISPR/Cas9 reagents) from the target LOF *BnaFAD2* allele (4-bp deletion; Okuzaki *et al*. 2018). In grain from the homozygous mutant plants, oleic acid content increased significantly and the C18 polyunsaturated fatty acids significantly decreased (Okuzaki *et al*. 2018). Recently, Huang *et al*. (2020) used hypocotyl transformation of cv. J9707, a semi-winter *B. napus*, with *Agrobacterium tumefaciens* to edit all three active *BnaFAD2* gene targets (Zhou *et al*. 2002). Transformation resulted in 108 PCR positive events (Huang *et al*. 2020). Of these, 16 yielded LOF *BnaFAD2* alleles; 3 were identified in *BnaC05.FAD2a*, 2 in BnaA01.FAD2b with the remaining 11 being in *BnaA05.FAD2a*, and no edits in *BnaC01.FAD2b* (Huang *et al*. 2020). Further, of the 11 *BnaA05.FAD2a* mutant lines, 6 had edits in both alleles (biallelic). Finally, two lines were heterozygous for mutations in each of *BnaC05.FAD2a* and *BnaA05.FAD2*a with two lines being heterozygous for mutations in each of *BnaA01.FAD2b* and with *BnaA05.FAD2a* being heterozygous in one line and having biallelic edits in the other (Huang *et al*. 2020). Off-target sites were assessed with none detected—all of which had 4 or 5 mismatches (Huang *et al*. 2020). Similar to what was observed by Okuzaki *et al*. (2018), in grain from the homozygous *BnaA05.fad2a* mutant plants, oleic acid content increased significantly (6–16%) and the C18 polyunsaturated fatty acids significantly decreased (Huang *et al*. 2020). In grain from the homozygous *BnaC05.fad2a* mutant plants, oleic acid content increased somewhat (about 5%) and the C18 polyunsaturated fatty acids decreased by a similar magnitude (Huang *et al*. 2020). Assessed as single seeds for a segregating population, an additive effect was observed when homozygous LOF mutations were combined for both *BnaA05.fad2a* and *BnaC05.fad2a* (Huang *et al*. 2020). In this population, 2 single seeds showed an oleic acid content exceeding >85% (Huang *et al*. 2020). Unlike Okuzaki *et al*. (2018), the reagent bearing transgene was not segregated away; therefore, mutations in the target genes in the progeny continued to accumulate (Huang *et al*. 2020).

Pod shatter reduction Seed pod opening and dispersal at harvest significantly affects yields with typical losses of 8–12% increasing upwards to 50% (Kadkol *et al*. 1986; Jaradat *et al*. 2014), if optimum harvest timing and practices, such as swathing, are not employed. A pod shatter reduction (PSR) trait offers farmers the advantages of flexibility for when to harvest the mature crop, reducing dockage for green seed (Hu *et al*. 2015), and peace of mind that grain will not be easily lost due to adverse weather, as well as savings in both time and fuel afforded from one less pass across the field. Additionally, more effective weed and disease control is provided since shattered grain can remain viable in soil for up to 17 yr, becoming weeds (volunteer plants) in the subsequent crops (Morgan *et al*. 2000; Jørgensen *et al*. 2007).

Liljegren *et al*. (2004) and Raman *et al*. (2014) investigated the genetic basis for pod shatter reduction and suggested a cascade of transcription factors was central to the phenotype. Using information related to pod structure elucidated through comparisons to *Arabidopsis*, it was determined that genes encoding two MADS-box transcription factors *SHATTERPROOF* (*SHP1*) and *SHATTERPROOF2* (*SHP2*), in addition two basic helix-loop-helix (bHLH) transcription factors *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*), influence shatter. In *Arabidopsis*, the SHP proteins induce the expression *IND*, *ALC*, and *SPATULA* (*SPT*) (Ferrándiz *et al*. 2000; Liljegren *et al*. 2000). Members of the *Brassicaceae* including *B. napus* and *Arabidopsis* have a seed pod (fruit) with two carpel valves joined to a replum, a septum that

divides the two carpels. A layer of two to three parenchymous cells between the valve edges and the replum forms the separation or dehiscence zone and can be identified because adjacent replum tissue contains the main vascular elements of the pod. Cell wall–degrading enzymes including cellulases and polygalaturanases are expressed in the dehiscence zone leading to its formation. While the seed pods are drying, they split along the dehiscence zone to shatter and release seed. Indehiscent mutants are completely indehiscent due to the absence of both lignified cells and the separation layer at the predetermined breaking point of the silique (Liljegren *et al.* 2004); *alc* mutants, however, only lack the separation layer (Rajani and Sundaresan 2001). Clearly, shatter resistance is a quantitative trait resulting from variation at multiple loci (Raman *et al.* 2014; Liu *et al.* 2016).

Braatz *et al.* (2017) used a CRISPR-Cas9 approach to inactivate the two *BnaALC* homologs, *BnaA07.ALCa* (BnaA07g12110D) and *BnaC07.ALCa* (BnaC07g16290D), upstream of the DNA-binding basic helix-loop-helix (bHLH) in canola. Arising from *Agrobacterium*-mediated transformation of 625 hypocotyl segments of SOSR cv. Haydn, over a period of 9–11 mo, 70 calluses were obtained of which 112 regenerated shoots with 4 bearing LOF frame shift alleles, two in each *BnaALC* homolog. When plants bearing the mutant *BnaALC* loci were assessed for shatter resistance, improvement of this phenotype was marginal, owing to high shatter resistance of the transformed cultivar itself (Braatz *et al.* 2018). Previously, this transformation method had most commonly been employed with the Westar cultivar (De Block *et al.* 1989).

Like Braatz *et al.* (2017), Zhai *et al.* (2019) used a CRISPR/Cas9 approach to independently inactivate the two *BnaIND* homologs, *BnaA03.IND* (BnaA03g27180D) and *BnaC03.IND* (BnaC03g32180D) as well as the two *BnaALC* homologs (described above). The authors accomplished this by hypocotyl transformation of the semi-winter OSR cv. J9707 using *Agrobacterium tumefaciens* (Zhou *et al.* 2002; Zhai *et al.* 2019). Of the more than 250 transformants for each of the two targets, >75% were PCR positive for the intended transgene (Zhai *et al.* 2019). Further characterization of these PCR positive transgenic lines identified 14 with confirmed LOF alleles in either *BnaIND* or *BnaALC*, including one single mutant *BnaA03.ind*, two *BnaC03.ind* single mutants, eight *Bnaind* double mutants, and three *Bnaalc* double mutants (Zhai *et al.* 2019). With the exception of one *Bnaind* mutant line, the remaining lines had loci with frameshifts leading to nonfunctional proteins. Similar to what was observed by Braatz *et al.* (2017), the PSR phenotype of the homozygous double mutant *Bnaalc* lines was similar to that of the parental control (Zhai *et al.* 2019). All homozygous double mutant *Bnaind* lines barely opened after their seeds matured and dried. For pod shatter resistance, these genes have partially redundant functions in canola pods with *BnaA03.IND* being more highly expressed and responsible for more of this quantitative effect than *BnaC03.IND* (Zhai *et al.* 2019). Developed using chemical mutagenesis, the dominant commercial pod shatter resistance trait in the North American market affects the *BnaIND* homologs.

Based on its function in *Arabidopsis*, the *JAGGED* (*AtJAG*) gene encodes a protein with a single C2H2 zinc-finger domain that controls the development of lateral organs (Dinneny *et al.* 2004). Zaman *et al.* (2019), with the aim of reducing pod shatter, used a CRISPR/Cas9 approach to generate LOF mutations in genes encoding the 5 *BnaJAG* paralogs (*BnaA02.JAG* (BnaA02g13870D), *BnaC02.JAG* (BnaC02g18270D), *BnaC06.JAG* (BnaC06g30050D), *Bna A07.JAG* (BnaA07g27150D), and *Bna A08.JAG* (BnaA08g24290D)). The authors used hypocotyl transformation of the semi-winter OSR cv. Zhongshuang 6 using *Agrobacterium tumefaciens* with the CRISPR/Cas9 vector having gRNAs designed to target all 5 loci and a separate construct targeting the *BnaA08.JAG* locus (Zaman *et al.* 2019). From plants regenerated from 106 callus lines, 41 were confirmed to be PCR positive for the *NPTII* selection marker gene. PAGE of annealed JAG amplicons identified 25 plants bearing at least one mutated paralog. Among these 25 mutants, 6 plants appeared to carry mutant alleles for all 5 *JAG* paralogs (Zaman *et al.* 2019). Line BnaJAG-5 was Sanger sequenced to confirm LOF loci in all 5 paralogs (Zaman *et al.* 2019). All 6 presumed pentuple mutants had a similar phenotype with pods having a cylindrical body that developed around the transmitting tract cells that guide the pollen tube from the stigma to the ovary (Zaman *et al.* 2019). The ovary appeared to be comprised of a number of undifferentiated cells (likely callus), without distinct differentiation of valves, replum, septum, and valve margins (Zaman *et al.* 2019). Pseudoseeds formed, but ultimately no viable seed was obtained from these lines. A separate line BnaJAG-33 was confirmed to have biallelic mutations in *BnaA08g24290D* (Zaman *et al.* 2019). This line appeared to be more resistant to pod shatter, with reduced lignification observed in the dehiscence zone; however, it had 50% wider and shorter pods with twisting valves along its whole length and only 10% of the seeds per pod compared with the WT (Zaman *et al.* 2019).

Conclusions

With publications beginning about 4 yr ago, in laboratories scattered around the globe, an increasing number of loci within the *Brassica napus* genome have been gene-edited using various technologies and reagent delivery methods. These efforts and their resulting publications have furthered basic research and offered opportunities for trait development in this important oilseed crop.

A few publications describe delivery of gene-editing reagents into protoplasts; however, plants bearing loss of function alleles developed in the papers herein cited were predominantly obtained using *Agrobacterium*-mediated transformation, principally of more easily transformed cultivars including Westar. Most applications of gene-editing in canola to date have involved delivery of DNA-based CRISPR/Cas9 editing reagents that are first integrated into the genome and expressed as a transgenic construct, and then segregated away by breeding as null segregants to leave only the desired LOF allele(s). However, this may be difficult to achieve in circumstances in which (1) DNA-based reagents may incorporate within the cut site or in a region tightly linked to the target allele or (2) in the case of complex multiplex/multi-target traits, in which large populations of at least 4^n (where n is the number of gene-edited loci) are required to obtain null segregants with these traits. Further, while DNA-based editing reagents allow the ability to enrich for transformants using selective agents such as kanamycin, hygromycin, or BASTA™, the time in culture required for efficient selection feeds a progressive gene-editing process that can lead to chimeras. For instance, Yang *et al*. (2017) observed that more than 30% of the transgenic T_0 plants analyzed were chimeric. This progressive editing may occur at a low frequency during the growth of the transgenic plants, and even in subsequent generations where the remaining WT alleles can be edited (Yang *et al*. 2018). To truly accelerate plant breeding, development of truly genotype independent methods for gene-editing within the range of *Brassica napus* cultivars is required.

A range of methods have been used to edit gene sequences in plants that have broadened the options to precisely fine-tune gene sequence including rare cutting endonucleases known as meganucleases, followed by engineered zinc finger nucleases, transcriptional activator-like effector nucleases (TALENs), and most recently, clustered regulatory interspaced short palindromic repeat (CRISPR)–associated protein (Cas) (CRISPR/Cas)—the latter which has become synonymous with the field of gene-editing (Chen and Gao 2014; van Eck 2020). Extending these editing tools, additional nucleases, such as Cas12a (Cpf1) with its differing PAM (protospacer adjacent motif) requirement compared to Cas9, have further expanded the available target sites for geneediting and new approaches, such as covalently linked activators, repressors, methylases, and base editors, as well as prime-editing, have broadened the options to precisely fine-tune gene sequence, including gene expression (Razzaq et al. 2019; Lin et al. 2020; van Eck 2020). As elaborated in this review article, most gene-editing applications in *Brassica napus* have involved making LOF alleles in various gene targets. Base-editing was deployed for a few targets enabling single letter changes within a few bases of the PAM site. These yielded a subset of expected letter changes. Further and precisely augmenting diversity within the *Brassica napus* genome will be enabled by extending past CRISPR/Cas9 to other CRISPR-associated systems as well as the application of editing technologies such as homology-directed gene-editing or prime-editing to enable the most precise and defined edits in this important oilseed crop.

As articulated throughout this review article, within the *B. napus* genome, many gene targets have at least one homolog from each of the A and C genomes with several targets, owing to the polyploidization events that led to the *Brassicaceae* being represented by 4, and even up to 8 independent loci (Chalhoub *et al*. 2014). Many traits such as yield and disease tolerance are complex and expected to require edits in multiple independent loci. Multiplex genome editing involves the simultaneous delivery of reagents to precisely edit multiple, related or unrelated, loci within a single cell and therefore within a single regenerated plant. Going further, the next step in the evolution of gene- editing technologies will be to combine gene-edited traits *de novo* as would be the case through introgression breeding, but with dramatically shorter timelines.

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