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New Flowering and Architecture Traits Mediated by Multiplex CRISPR-Cas9 Gene Editing in Hexaploid *Camelina sativa*

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Abstract: Adapting plants to sustainable cropping systems is a major challenge for facing climate change and promoting agroecological transition. *Camelina sativa* is an emerging oilseed crop species with climate-resilient properties that could be used in double-cropping systems, in particular as a summer catch crop. The availability of early-flowering camelina is essential in such cropping systems to allow full completion of the growth cycle during summer. Targeted induced gene variation (TIGV) was used in camelina on several flowering repressor genes identified in Arabidopsis to obtain early-flowering lines. Multiplex editing of 15 target genes representing *FLOWERING LOCUS C*, *SHORT VEGETATIVE PHASE*, *LIKE HETEROCHROMATIN PROTEIN 1*, *TERMINAL FLOWER 1* and *EARLY FLOWERING LOCUS 3* induced combinatorial mutations that were screened for early-flowering phenotypes. Certain mutants showing a stable early-flowering trait after five generations also presented additional phenotypes: determinate flowering, shorter stature and/or basal branching. Different combinations of mutations had a positive or negative impact on yield. This work demonstrates that efficient multiplex CRISPR is achievable in hexaploid plants like camelina, providing valuable genetic diversity for better selecting lines adapted to new cropping systems.

Keywords: CRISPR; flowering; multiplex; agroecology

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1. Introduction

Sources of genetic diversity have always constituted the raw materials for plant breeding; however, for certain crop species, the existing diversity is a major limiting factor in crop improvement. We have used a multiplex CRISPR strategy to create traits of agronomic interest in camelina (*Camelina sativa* (L.) Crantz), a re-emerging oilseed crop species in the Brassicaceae family in which the existing diversity is limiting (for a review, see [1–4]). Camelina, also known as false flax, is closely related to well-known wild species, including the intensively studied model species *Arabidopsis thaliana* and the widespread weed *Capsella bursa-pastoris*, shepherd's purse [5]. Over the millennia following its domestication, camelina was widely grown in the cooler regions of Europe; however, starting at the end of the 19th century, it was gradually replaced by higher-yielding oilseed crops such as rapeseed [6]. Nonetheless, during the 20th century, camelina continued to be cultivated on a small scale, essentially for the production of oil for human consumption. Because of this century of neglect, camelina has undergone relatively little improvement by plant breeders; thus, it should benefit greatly from the combined efforts of plant breeding and advanced techniques of modern biotechnology.

Camelina has also attracted attention because of the inherent agronomic properties of the crop. In particular, it can be grown on marginal soils, thriving as well in the dry regions of the high plains of North America [3] as in the well-watered climate of Ireland [7]; since it appears to be resistant to pests and pathogens of other Brassicaceae crops, it requires few or no agrochemical treatments. Camelina's short life cycle has also led to suggestions that it could be used in double-cropping systems. This was first shown by field trials in which a

winter camelina crop was followed by a spring-planted soybean [8]; this double-cropping system has been shown to have quite positive agro-ecological features [9]. It has also been suggested that the converse double-cropping system should be possible, in which a winter crop would be followed immediately by camelina sown in late spring or early summer [10]. However, when this strategy of using camelina as a summer catch crop was tested by farmers at several locations in northern France, they were confronted with two problems: weeds were often serious competitors, and the camelina did not reach sufficient maturity in time to be harvested [11]. This was also the case when double-cropping with spring camelina was tested over several years at the Versailles INRAE experimental farm (M. Tepfer, unpublished).

Camelina has also attracted considerable attention as a model crop for translational research in plant biotechnology, since genetic transformation and gene editing are attractive tools for overcoming the crop's limited genetic diversity. Furthermore, camelina is easily transformed by *Agrobacterium* flower dip [12], has a short life cycle in the greenhouse, and its close genetic relationship to the model species Arabidopsis means that genes equivalent to those in Arabidopsis are readily identified in the camelina genome, for which a reference sequence is available [13]. The sequencing of 222 natural accessions and breeding lines demonstrated that important quantitative trait loci (QTL) could be identified for important agronomical traits such as seed size, oil content, fatty acid composition, and flowering time, albeit with a lower level of diversity compared to other domesticated crops [14].

However, in recent years, biotechnology applications have been used to create novel camelina traits, mostly for oil composition, beyond what could be achieved by traditional breeding. For instance, transgenic camelina that produces oil that is closely equivalent to fish oil has been created [15,16]; CRISPR-Cas9 knockout of one or more of the three camelina *FAD2* genes generated genotypes in which the oil is enriched in oleic acid to varying degrees [17], and camelina has been used as a platform for producing oil-soluble terpenes [18] or insect pheromones (https://www.eurekalert.org/pub_releases/2021-01/ii-gpg011421.php accessed on 17 June 2022).

The genome editing tools that have been developed over the past decades have revolutionized biomedical research, and more recently have been applied to crop improvement [19]. In most cases, agronomic traits have been created by targeting a small number of genes, most often by inducing knockout mutations. However, there are cases where multiplex CRISPR has made it possible to modify genetically complex traits, such as in tomato [20], kiwifruit [21], or cotton [22]; for a review, see [23]. We have used a highly multiplexed variant of the targeted induced genetic variation (TIGV) CRISPR strategy [24] to create novel traits of growth and development in camelina that are expected to be of agronomic interest, particularly in view of improving the integration of camelina in sustainable agricultural practices, such as novel cropping systems.

The primary purpose of this study was to create camelina genotypes with a shorter life cycle, associated with other developmental traits that may have beneficial effects in the field. The flowering transition, in which the shoot apical meristem switches from producing leaves and stems to producing flower buds, is a major determinant of the length of the plant life cycle. In the thirty years since the pioneering work of Koornneef [25] described a collection of Arabidopsis flowering-time mutants, the genetic control of the flowering transition has been extensively studied, particularly in Arabidopsis, as best exemplified by the complexity of the Wikipathway dedicated to it [26]. One constant that emerges from these studies is the central role played by the *FLOWERING LOCUST* (*FT*) gene [25], which acts as an integrator of numerous environmental signals, and encodes the signal molecule that is transported from leaves to the shoot apical meristem where it leads to changes in the expression of the floral meristem identity genes that determine flower development [27]. Thus, for this study, three camelina orthologs of genes that are direct repressors of *FT* in Arabidopsis were chosen for CRISPR-mediated inactivation: *FLOWERING LOCUS C* (*FLC*) [28] *SHORT VEGETATIVE PHASE* (*SVP*) [29], and *LIKE HETEROCHROMATIN PROTEIN 1* (*LHP1*) [30,31]. Many of the flowering-time genes have pleiotropic effects,

some of which may be of agronomic interest. For instance, mutations in *SVP* increased basal branching [32], which in camelina may confer an advantage in competition with weeds. Two additional genes were selected because of their dual functions. Inactivation of *TERMINAL FLOWER 1* (*TFL1*) in Arabidopsis leads to early flowering [33], but also to a determinate growth habit, which may favor the synchrony of the end of flowering and, thus, better synchronize seed maturation. Inactivation of *EARLY FLOWERING LOCUS 3* (*ELF3*) causes early flowering in Arabidopsis [34], but also modulates thermomorphogenesis, allowing potential adaptation to high temperature [35,36].

Here, we show that applying the TIGV strategy to five flowering-repressor genes *FLC, SVP, LHP1, TFL1* and *ELF3* in camelina leads to a varied set of mutants with flowering precocity combined in some cases with determinate flowering, basal branching, and/or short stature. The sequencing of several lines at the T5 generation identified the mutations present in the 15 target genes.

2. Materials and Methods

2.1. Construction of Binary Vector pFlo6 Bearing the Genes Necessary for CRISPR-Cas9 Mutation of the Five Target Genes

A vector for *Agrobacterium*-mediated transformation, pFlo6, was built in successive steps using GoldenBraid II technology [37,38]. For each of the five genes targeted for mutation, two sgRNAs that target conserved sequences of the three homeologs were designed. The sgRNAs with the highest quality scores and lowest off-target probability were designed using CRISPOR software (http://crispor.tefor.net/ accessed on 17 June 2022) [39], and sites close to the 5′ end of the target genes were preferred, in order to increase the probability that frameshift mutations would totally inactivate the protein's function.

The individual transcriptional units for expression of the ten sgRNAs controlled by the pU6–26 promoter were assembled in successive GoldenBraid steps. In parallel, an expression cassette with the plant-optimized Cas9 coding sequence (pcoCas9) between the parsley ubiquitin 4 (PcUbi4_2) promoter and the pea RbcsE9 terminator was assembled, and also one in which the fluorescent marker DsRed coding sequence was placed between the Cassava vein mosaic virus (CsVMV) promoter and the *A. tumefaciens* Nos terminator. All these elements were assembled together in the pCAMBIA binary vector to create pFlo6. The presence and order of the 10 sgRNA transcriptional units were confirmed in *E. coli* and *A. tumefaciens* two by two (sgRNA1 and sgRNA2 for each gene) by PCR using the forward primer of ELF3 sgRNA 1 (i.e., LG1051 for_ELF3a) with the reverse primer of the ELF3 sgRNA 2 (LG1056 for_ELF3b), then using the forward primer of the ELF3 sgRNA2 (LG1055 for_ELF3b) with the reverse primer of LHP1 sgRNA1 (LG1082 sgRNA_LHP1a) and so on until the target was in position 10 (sgRNA_SVPb). The positions of the sgRNA transcriptional units are indicated in Figure 1 and the primers are indicated in Table S1.

2.2. Plant Growth Conditions and Plant Transformation

Plants were grown in the greenhouse with a 16-h daylength supplemented with artificial light when necessary, 25 °C day and 22 °C night temperatures, and 40% relative humidity. T1 plants were grown in 14 cm pots, and plants in the following generations in ca 300 mL pots in 24-pot trays. Multipots were watered as needed by subirrigation, flooding the table on which they were grown until the substrate was saturated, then drained completely. After annotation for the date of flowering was finished, cords were strung the length of the tables to prevent lodging as the plants grew in height. When the seed pots on the lowermost branches began to yellow, watering was gradually decreased over a week to allow seed maturation, then ceased to allow the plants to dry.

Floral dip transformation of camelina (*C. sativa* cv Céline) plants with pFlo6 was performed as described [40]. The only additional feature was that the penetration of the *Agrobacterium* suspension was enhanced using the purpose-designed vacuum chamber described in [4].

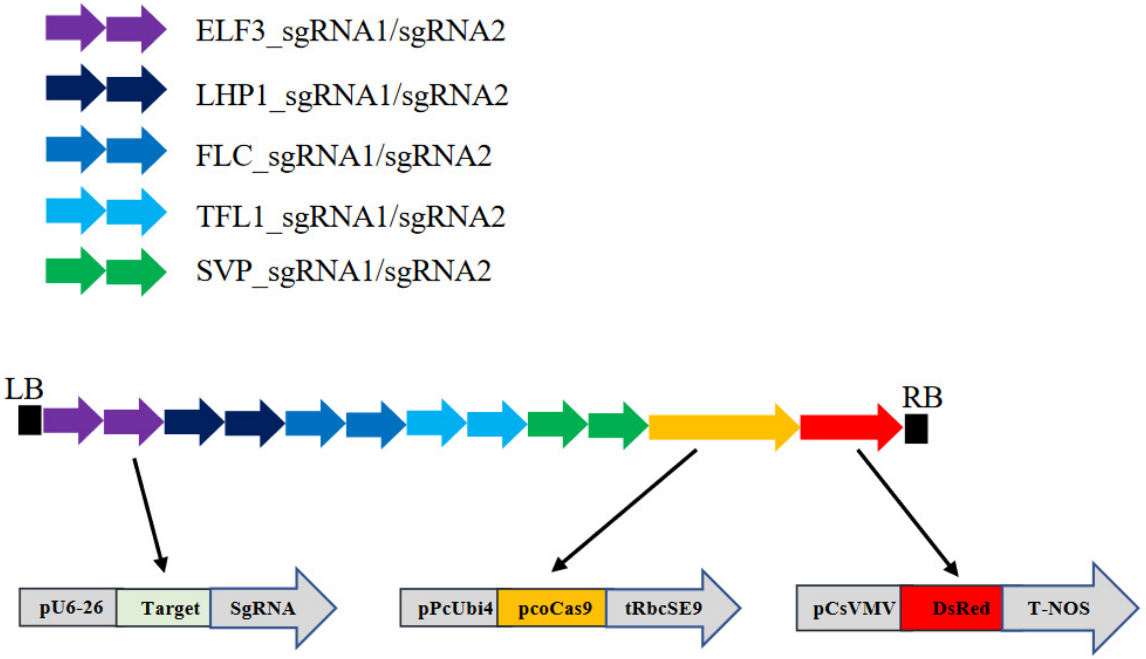

Figure 1. Organization of the T-DNA used to CRISPR/Cas9 mutagenize camelina via *Agrobacterium* floral dip. LB and RB represent the left and right borders of the T-DNA, between which are shown, from left to right, the ten genes encoding sgRNAs, the pcoCas9 gene, and the DsRed marker gene.

2.3. Plant Nomenclature

In order to be able to follow the genealogy of each plant, starting in the T2 line, they were numbered according to their position in the multipots in which they were grown. In order to distinguish plants that were DsRed-positive for a single generation from those that were so for two generations, the former were numbered 1–24, and the latter, 101–124 in the T2 generation. At each following generation, an additional number was added. Thus, for example, the plant named 6-1-104-40-22-20 was: a T5 descendant of plant number 1 transformed with the Flo6 T-DNA; in the T2 generation, plant 4 among the DsRed-positive plants; and plant 40, 22, and 20 in the successive generations. Simplified names were, nonetheless, used in the text, and the correspondence between names and pedigrees is shown in Table S2.

2.4. Phenotype Analysis

As shown in Figure 2A, a date tag was attached to each plant when the first flower opened, which corresponds to stage 60 of [41]. At the end of the plant growth cycle, when the plants were completely dry, individual plants were removed from the multipots, and the following parameters were noted for each: date of flowering, plant height, presence/absence of determinate growth, and number of branches emerging from the most basal 10 cm of the main stem. Seeds were harvested from each plant, and the yield (g of seeds/plant) was determined.

Seeds borne by the T5 plants were photographed using an Axiozoom (Zeiss), and images were segmented by 'distance transform watershed' as implemented in the MorpholibJ plugin [42]. The quantification of the areas was extracted as described in [43]. R software was used to run the wilcoxon.test for plant height, number of basal stems, and seed yield; however, the Student test was used for seed size.

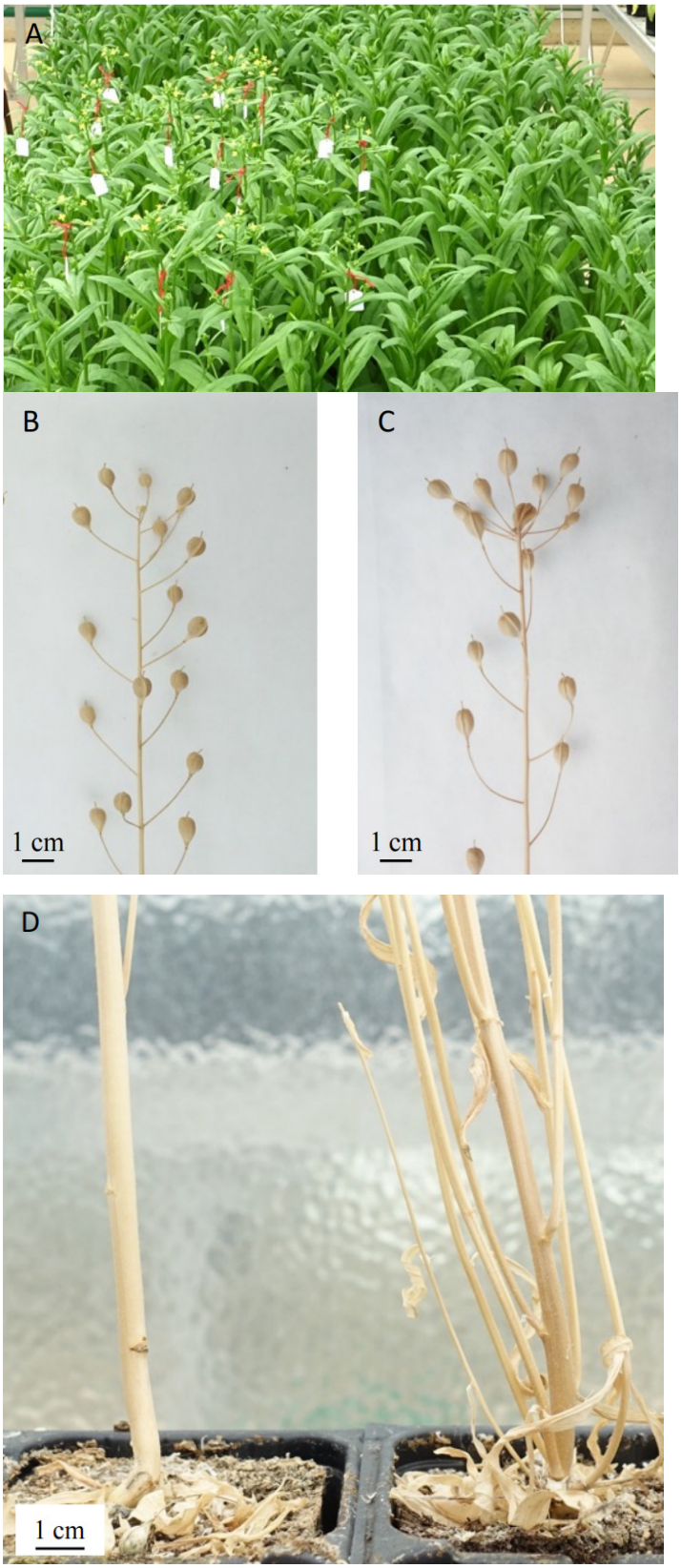

Figure 2. Novel phenotypes observed in the T3, T4, and T5 plants. Overview (**A**) of an early-flowering progeny (**left**) and controls (**right**) shown 30 days after sowing. Main branch of a mature control plant (**B**), and a plant showing a strong determinate growth phenotype with a terminal seed pod (**C**). In (**D**) are shown a control plant (**left**), and one showing abundant basal branching (**right**).

2.5. Genotype Analysis

The total DNA of camelina was extracted from 50 mg of ground frozen tissue (young leaves) using an extraction buffer (200 mM TrisHCl pH 7.5, 250 mM NaCl, 25 mM EDTA, 0.5% SDS). After isopropanol precipitation and 70% ethanol cleaning, the DNA was dissolved in 100 µL of TE buffer. PCR genotyping (recombinant Taq polymerase, ThermoFisher Scientific, Foster city, CA, USA) was performed using 1 µL of extracted DNA and subgenome-specific primers amplifying the targeted loci (Table S5). The quality of the DNA samples was evaluated using primers targeting the *C. sativa EF1* gene. Sequence analysis was based on Sanger sequencing (Genoscreen, Lille, France) of PCR fragments for each targeted locus of the five target genes. Sequences were aligned by CodonCode Aligner V5.1.5 from LI-COR, using DNA of *C. sativa* cv Céline as a reference template. Sequences of heterozygous plants were analyzed using ICE software (https://ice.synthego.com accessed on 17 June 2022). For the *SVP* gene, as specific amplification of the SG-2 subgenome could not be obtained, PCR products using primers amplifying SG-1 and SG-2 copies were cloned into the pCR2-TOPO TA vector (ThermoFisher Scientific, USA), followed by Sanger sequencing (Genoscreen, France). The genotyping primers used are listed in Table S3, and the sequences are given in Table S4.

3. Results

3.1. Overall Experimental Strategy

Since the camelina genome is hexaploid, with three closely related subgenomes [13,44,45], a total of 15 camelina homeologs corresponding to the Arabidopsis *FLC, SVP, LHP1 TFL1* and *ELF3* genes were identified. All the homeologs were targeted simultaneously, since their different expression levels [13,46] might reflect some degree of sub- or neo-functionalization (Table S5). This strategy generated a combinatorial library of mutants, which was screened for phenotypes of interest, mainly early flowering, determinate flowering, and modification of stem architecture. The progeny produced by single-seed descent was screened over several successive generations, obtaining T4 lines approaching phenotypic homogeneity, indicating a high level of homozygosity of the mutations. The CRISPR target sites in nine lines of interest were sequenced in T5 generation plants.

3.2. Genetic Transformation and Production of Construct-Free Segregants

The sgRNAs were designed in regions of sequence identity shared by the three homeologs for each of the five target genes. The sgRNAs with the highest quality scores and lowest off-target probability were designed using CRISPOR software (http://crispor.tefor.net/ accessed on 17 June 2022) [39]; sgRNA targets close to the 5′ end of the open reading frame (ORF) of the genes of interest were preferred in order to increase the probability of inactivating frameshift mutations. Two sgRNAs for the three homeologs of each gene were selected for a total of 10 sgRNAs for the 15 targets (Figure 1 and Table S6). The sgRNA transcriptional units were included in a single T-DNA that also comprised Cas9 and a DsRed marker gene (Figure 1 and Figure S1). The plants were transformed by floral dip and enhanced by vacuum infiltration [4]. Fourteen DsRed-positive T1 transformants were selected, and 24 DsRed-positive and 24 DsRed-negative T2 segregants were grown for six T1 (Figure S2). Through the T3 to T5 generations, only DsRed-negative plants were included, i.e., the progeny of the DsRed-negative T2 s, as well as the DsRed-negative segregants of the DsRed-positive T2 s. Thus, plants bearing the CRISPR-Cas9 transgenes for either one or two generations were included in the study (Figure S2). A total of 87, 34, and 15 lines were screened, respectively, in the T3, T4, and T5 generations, with 24 plants per family, except for T3 where 48 plants per family were screened.

3.3. High Plant Density Phenotype Screen

Except for the initial transformed individuals (T1 generation), plants of all the following generations were grown in 24-pot multipot trays. This had two advantages compared to plants grown in individual large pots: (1) large numbers of individuals could be grown

using limited homogeneous greenhouse space (for example, more than 4000 T3 plants were grown simultaneously); and (2) the plants more closely resembled ones grown in the field. Concerning the latter point, plants grown in large pots in the greenhouse branch abundantly at the base, producing several racemes/plant [41,47], whereas in the field and in multipots, each plant has a single raceme that only branches distally.

Since high density culture in multipot trays led to the intermingling of inflorescence branches of adjacent plants, it raised the potential problem of outcrossing between different plants by physical contact. If there were extensive outcrossing, this would compromise the single-seed-descent strategy. We therefore tested this hypothesis by growing, in association with several generations of selfing of the edited plants, a homozygous plant expressing either a GFP or a DsRed transgene [40] in the central positions of a tray of 24 control plants. The seeds were harvested from individual plants and were examined for DsRed or GFP fluorescence. In all cases, there was at most one fluorescent seed/tray borne by a non-fluorescent plant, showing that crossing between plants was negligible in these conditions (data not shown). These results are compatible with the very low outcrossing rates observed in the field in Canada [48,49].

3.4. Selection of Early Flowering Mutants

Early flowering plants were identified as early as in the T1 generation (Table S3). At each generation, individual plants were tagged the day the first flower opened (Figure 2A), corresponding to stage BBCH 60 [41]. Interestingly, other features including late flowering, basal branching, determinate flowering, and short stature were noted as they appeared through successive generations (Table S7). Early-flowering individual plants were selected and their progeny were grown up to the T4 generation; however, plants with undesirable phenotypes, such as late flowering and extreme dwarfism, were not carried on to the T5 generation, and were not further characterized. As shown in Figure 2B,C, several lines displayed a distinctive type of determinate growth, in which the last internodes are shortened, and branches end in a terminal flower reminiscent of Arabidopsis *tfl1* mutants [35], suggesting the possible inactivation of one or more of the camelina *TFL1* homeologs. In addition, certain lines developed additional basal branches that appeared approximately simultaneously with the bolting of the main stem (Figure 2D).

3.5. Phenotyping of T5 Mutants

T5 plant families were phenotyped individually in more detail at harvest. For each plant, the date of flowering, plant height, number of basal branches, determinate vs. indeterminate growth, and seed yield were evaluated.

Figure 3 shows the time course of flowering in 15 selected T5 families. Four lines (B1-2, A40-24, C2-19, and B20-2) were particularly precocious, since they flowered a week or more earlier than the non-edited Céline control, and they were the progeny of the earliest flowering lines of the T4 generation (not shown). The A1-4 line was the least precocious, flowering about two days earlier than Céline.

The two most precocious lines, B1-2 and C2-19, displayed a strong *tfl1*-like determinate growth habit early in inflorescence development. Several other lines also showed *tfl1*-like determinate growth, but at a later stage (B33-8, C2-23, C16-4, C16-9, C27-4). Lines A4-12, A4-19, and B1-11 segregated for this feature, with 6/24 plants with determinate growth for the former two, and 8/23 for the latter. All of the early-flowering T5 families were distinctly shorter in stature compared to the controls, and this was particularly notable for the lines B1-2 and C19 (Figure 4A). Basal branching was also observed first in the T3 generation and confirmed in the T5 generation for lines A4–12, A4–19, and A1–4. These lines showed 4–5 basal branches, whereas all the other lines including Céline had only a single stem (Figure 4B). Combinatory phenotypes were observed among the early-flowering lines, with the association of determinate growth and/or basal branching phenotypes (Table 1).

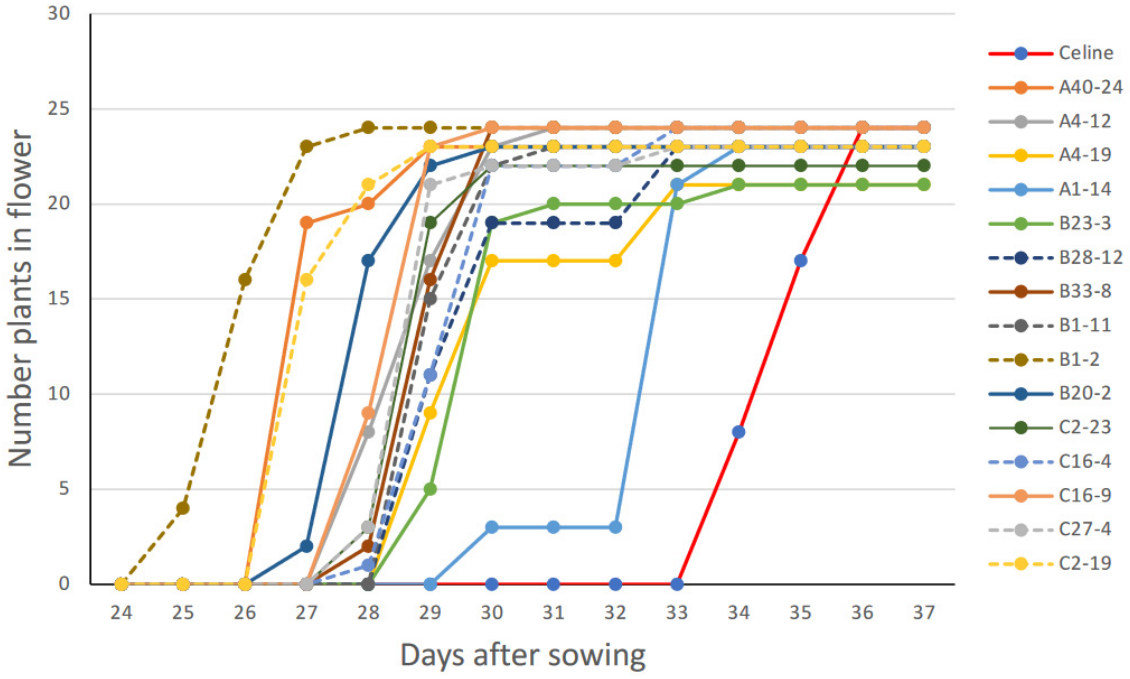

Figure 3. Time to flowering in the T5 families. Plants were grown in 24-pot multipots. A date tag was attached to each plant on the day when the first flower opened. The date of flowering was noted for each plant when they were harvested.

Table 1. Summary of phenotypes of the T5 family.

T5 Line	Early Flowering	Shorter Stature	Determinate Growth	Basal Branching
Céline				
B1-2 *	+++	+++	+++	−
A40-24 *	++	++	−	−
A4-12 *	++	++	−/++	+++
A4-19 *	++	++	−/++	+++
A1-14 *	+	++	−	+++
B23-3 *	++	++	−	−
B28-12	++	++	−	−
B33-8	++	++	++	−
B1-11	++	++	−/++	−
B20-2				
C2-23	++	++	++	−
C16-4	++	++	++	−
C16-9	++	++	++	−
C27-4	++	++	++	−
C2-19 *	+++	+++	+++	−

+, ++ and +++ correspond to the intensity of the particular phenotype feature: +++ being the strongest; − indicating no difference with the control line; and −/++ indicating a segregating phenotype. Asterisks (*) indicate lines in which the target sites were sequenced in one or two T5 individuals.

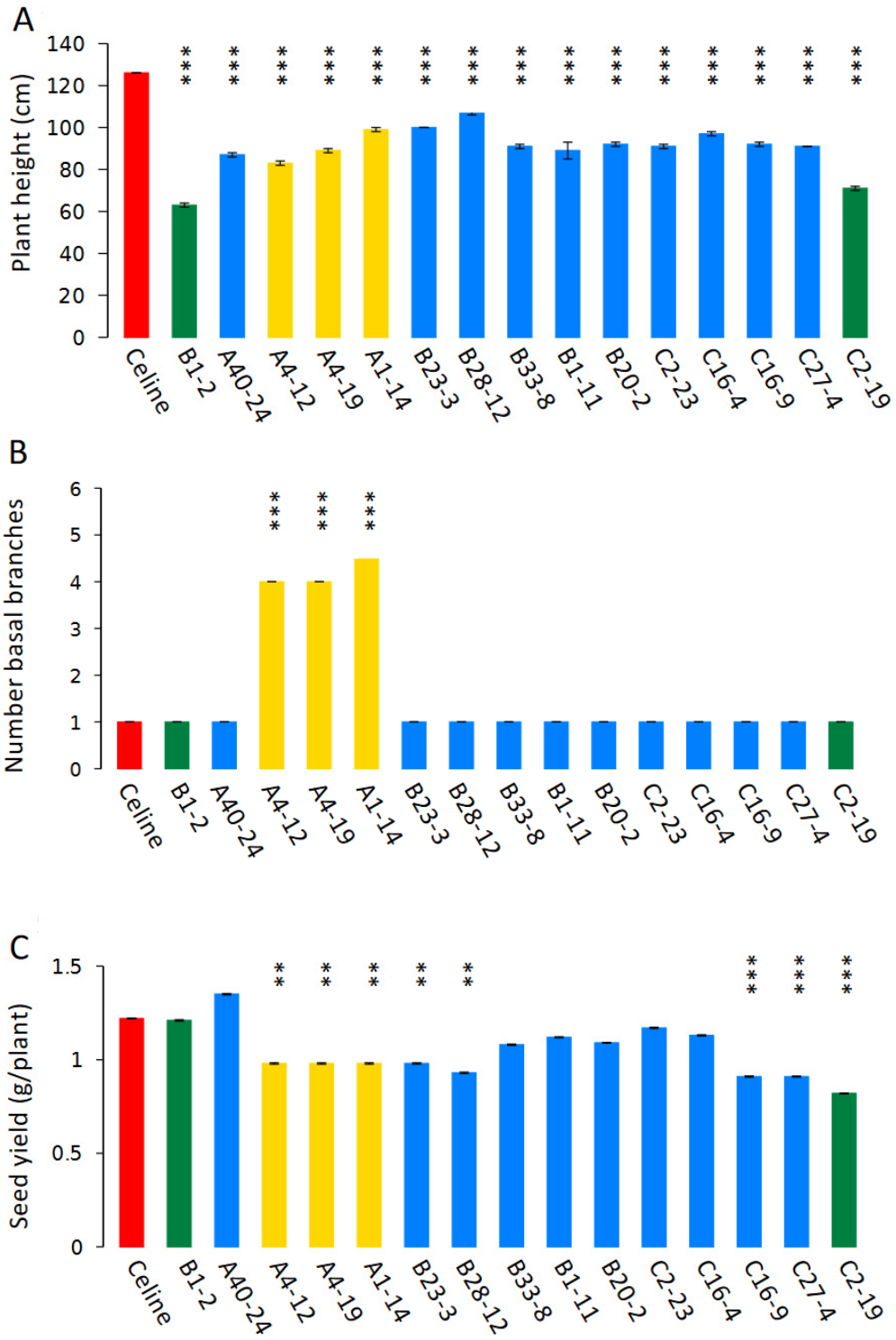

Figure 4. Phenotype of the T5 families. Plants were grown in 24-pot multipots. Final plant height (**A**), number of basal stems/plant (**B**), and seed yield/plant (**C**) were noted for each plant at harvest. The values shown are means +/− SEM. In (**A**–**C**), $n = 24$. *** $p < 0.001$; ** $p < 0.01$.

Since in some crops there can be a tradeoff between precocity and yield, the seed yield/plant was determined in each T5 family (Figure 4C). Remarkably, although certain lines had a lower yield than the control line, several of the earliest lines showed no significant yield penalty, including one with a determinate growth habit (B1-2), and one with a normal indeterminate growth (A40-24). Early flowering in *tfl1* Arabidopsis mutants was also associated with larger seeds [50]. Since *TFL1* was one of the targeted genes, the seed

area was determined for several lines: two with strong *tfl1*-like determinate growth (B1-2 and C2-19); three with increased branching (A4-12, A4-19, and A1-14), of which the former two displayed a late-onset *tfl1*-like phenotype; and one with earlier flowering without these two other traits (A40-24) (Figure S3). Indeed, the two families with strong *tfl1*-like determinate growth, B1–2 and C2–19, showed larger seeds, but the effect was much less than that described in Arabidopsis *tfl1* mutants; an increase of 14% was observed in line A40–24, versus more than 25% in Arabidopsis *tfl1* mutants [50]. Furthermore, larger seeds were also observed in line A40–24, and in one line with increased basal branching, A1–14, neither of which had a *tfl1*-like phenotype.

3.6. Genotypes of T5 Lines

Since several lines were phenotypically equivalent and siblings with potentially the same alleles, the determination of the CRISPR-Cas9 mutations was carried out only in representative families: two early lines with a strong determinate habit (B1-2 and C2-19), two early lines with indeterminate growth (A40-24 and B23-3), one line with moderate precocity and basal branching (A1-14), and early lines with basal branching with or without determinate growth (A4-12 and A4-19). Since determinate growth was still segregating in the latter two lines, the CRISPR target sites of two plants of each were sequenced, whereas single plants representing the other families were studied. As shown in Table 2, there was considerable variation among the lines studied concerning the number of target sites mutated (5–14 sites/line) and the number of mutated genes (5–10 genes/line). Most of the mutations were typical for Cas9, with usually a single-nucleotide insertion or deletion of a small number of nucleotides. In a few cases, deletions were accompanied by one or two single-nucleotide changes (snp). In only one case, a deletion including both target sites was observed (123 nucleotides); however, this was not a precise deletion between the predicted CRISPR cleavage sites.

Table 2. Summary of genotypes of the T5 individual plants.

Gene	Accession	sgRNA	T5 Plant								
			A40-24-25	A4-12-25	A4-12-26	A4-19-25	A4-19-26	A1-14-25	B23-3-25	B1-2-25	C2-19-25
SVP	Csa16g044290	1	WT	WT	WT	WT	WT	WT	WT	WT	WT
		2	WT	WT	WT	−7/WT	WT	WT	WT	+T	−41
	Csa07g052630	1	WT	WT	WT	WT	WT	WT	WT	WT	WT
		2	WT	WT	WT	WT	WT	+C	WT	+C	WT
	Csa09g086860	1	WT	WT	WT	WT	WT	WT	WT	WT	WT
		2	+C	−7/WT	WT	WT	WT	WT	−34	−5	−4
FLC	Csa13g011890	1	WT	WT	WT	WT	WT	WT	WT	WT	WT
		2	WT	WT	WT	WT	WT	WT	WT	WT	WT
	Csa08g054450	1	WT	WT	WT	WT	WT	WT	WT	WT	WT
		2	+T	WT	WT	WT	WT	WT	WT	WT	WT
	Csa20g015400	1	WT	WT	WT	WT	WT	WT	WT	WT	WT
		2	WT	WT	WT	WT	WT	WT	WT	WT	WT
LHP1	Csa13g020480	1	−20	+C	+C	+C	+C	WT	WT	+A	+C
		2	WT	−ACG	−ACG	−ACG	−ACG	5	+A	WT	WT/−1
	Csa08g009910	1	WT	WT	WT	WT	WT	WT	+A	+A	+A
		2	WT	−4	−4	−4	−4	−4	WT	−10/snp	+T
	Csa20g025860	1	WT	WT	WT	WT	WT	−123	WT	+C	-G
		2	WT	WT	WT	WT	WT	−123	−A	WT	WT
TFL1	Csa13g003940	1	+T	+T	+T	+T	+T	+A	WT	+A	+T
		2	+A	WT/−2	WT/−2	WT/−2	WT	WT	WT	+C	+T
	Csa08g060650	1	WT	WT	WT	WT	WT	WT	+A	+T	+C
		2	WT	WT/+T	+A	WT/+T	WT/+T	+A	WT	−4	−4
	Csa20g005030	1	+G	WT	WT	WT	WT	WT	WT	WT	CD
		2	WT	−4 & 2snp	−4 & 2snp	−4 & 2snp	−4 & 2snp	WT	WT	−4	CD
ELF3	Csa16g047550	1	WT	WT	WT	WT	WT	WT	WT	−T & snp	−C
		2	WT	WT	WT	WT	WT	WT	WT	WT	WT
	Csa07g056950	1	WT	WT	WT	WT	WT	WT	WT	WT	WT
		2	WT	WT	WT	WT	WT	WT	WT	WT	WT
	Csa09g092140	1	WT	WT	WT	WT	WT	WT	WT	WT	WT
		2	WT	WT	WT	WT	WT	WT	WT	WT	WT

For each homeolog, sequenced alleles are presented for the two sgRNAs positions (1 or 2): wild-type (WT), insertions or deletions (− or + nucleotides), single nucleotide polymorphism (snp), and complex deletions (CD). The dark grey shading represents a homozygous frameshift mutation at one or both sgRNA sites (gene inactivation). Furthermore, the light grey shading represents a heterozygous frameshift mutation at one or both sgRNA sites or a homozygous frameshift mutation at one or both sgRNA sites, but with the possible re-establishment of the ORF by the mutation at the second sgRNA site (possible gene inactivation).

4. Discussion and Conclusions

This project succeeded in generating camelina lines that in the greenhouse flowered considerably earlier than the controls, and in some cases displayed other potentially useful

features, notably determinate growth and increased basal branching. A recent study incorporating field evaluation of camelina accessions and genome-wide association mapping of important agronomical QTLs indicated that days-to-flowering and days-to-maturation are positively correlated, confirming that early flowering is a reasonable proxy for a shorter life cycle [14]. The same study also suggested that it would be possible to breed early flowering without compromising seed yield. However, the genetic diversity of existing camelina populations is relatively low [14,51], which could limit breeding approaches. Editing is therefore an interesting alternative strategy for the creation of new alleles expanding existing genetic resources.

It is of note that among the lines that emerged in the screen for early flowering, there were clear differences in the target genes that were mutated. At least one of the three homeologs of *LHP1* and *TFL1* were mutated in all the early-flowering lines, while *SVP, FLC,* and *ELF3* were only occasionally mutated. These differences may be due to possible differences in sgRNA effectiveness and/or due to phenotyping biases. Interestingly, early-flowering mutants were all shorter than the controls and had at least one homeolog of *LHP1* and *TFL1* mutated, suggesting these two genes might also be involved in plant height. In contrast, no mutations in *SVP, FLC*, and *ELF3* could be observed in these shorter and early-flowering lines, excluding the involvement of these genes in plant stature, at least in those lines.

Concerning increased basal branching, mutations in *SVP* were reported to cause this phenotype in Arabidopsis [14,51]; however, no mutations in the *SVP* genes were observed in the highly branched camelina lines.

Concerning determinate growth, three categories of lines were obtained: two T5 lines (A40-24 and B23-3) did not display a determinate phenotype in that generation or any previous one; two lines (B1-2 and C2-19) expressed determinate growth early in development; and the remaining lines switched to determinate growth only later in development. The *TFL1* genes are the most likely candidates for causing the determinate phenotype; however, there was no apparent correlation between the number and nature of *TLF1* mutations and early vs. late determinate growth. Furthermore, one TLF1 homeolog was mutated in the two lines that did not display a *tfl1*-like phenotype in the T5 or any previous generations. It remains that these different phenotypes could result from specific gene dosage effects associated or not with specific alleles or even the neofunctionalization of some homeologs.

Altogether, the TIGV strategy proved to be effective for creating new phenotypes associated with combinations of alleles in the homeologs of the five target genes. It remains now to evaluate these lines under real agronomical conditions. Indeed, the camelina life cycle is longer in the field, and it will be important to determine whether these edited lines are still early-flowering in the field with same time shift (7–10 days relative to the controls), or whether the precocity will be proportional to the life cycle (~25%). The precocity of maturation would be an important trait to adapt camelina as a summer catch crop in interculture. Similarly, field trials will determine if increased basal branching could enhance competition against weeds, a major limitation in interculture; and whether determinate growth could improve the simultaneity of pod maturation and, therefore, the harvest seed homogeneity. If the early-flowering phenotype is confirmed in the field under the usual spring sowing protocol, the following step will be the evaluation of the adaptation of these lines to double-cropping scenarios, in which the short-cycle camelina would be sown immediately after the harvest of a winter crop.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12081873/s1, Figure S1: Structure of binary plasmid carrying TDNA with sgRNA, Cas and DsRED marker; Figure S2: Genealogy of edited camelina populations; Figure S3: Phenotype of T5 plants; Table S1: primers used for T-DNA construct validation; Table S2: Line and plant naming and pedigree; Table S3. Primers used for amplification and sequencing; Table S4. Primer sequences; Table S5: Compilation of transcriptomic data concerning the

genes targeted for knockout; Table S6: Floraison-6 target genes, subgenomes, sgRNAs used to mutate genes; Table S7: Progressive appearance of phenotypes.

Author Contributions: M.T., J.-D.F. and F.N. designed the research; L.G. assembled the transgenes; Y.B. and M.T. transformed the plants; M.T. carried out plant phenotyping; Y.B., A.G.-D. and T.F. genotyped the target site mutations with the help of L.G.; E.B. carried out the seed size analysis; M.T. wrote the manuscript with contributions from all the authors. All authors have read and agreed to the published version of the manuscript.

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