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Removing the major allergen Bra j I from brown mustard (*Brassica juncea*) by CRISPR/Cas9

Juvenal Assou[1,†], Dingbo Zhang[2,†], Kristian D. R. Roth[3], Stephan Steinke[3], Michael Hust[3], Thomas Reinard[2], Traud Winkelmann[1]* and Jens Boch[2]*

[1]*Institute of Horticultural Production Systems, Leibniz Universität Hannover, Hannover, Germany,*
[2]*Department of Plant Biotechnology, Institute of Plant Genetics, Leibniz Universität Hannover, Hannover, Germany, and*
[3]*Department of Biotechnology, Institute of Biochemistry, Biotechnology, and Bioinformatics, Technische Universität Braunschweig, Braunschweig, Germany*

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*For correspondence (e-mails traud.winkelmann@zier.uni-hannover.de [T.W.]; jens.boch@genetik.uni-hannover.de [J.B.]).
†These authors contributed equally to this work.

SUMMARY

Food allergies are a major health issue worldwide. Modern breeding techniques such as genome editing via CRISPR/Cas9 have the potential to mitigate this by targeting allergens in plants. This study addressed the major allergen Bra j I, a seed storage protein of the 2S albumin class, in the allotetraploid brown mustard (*Brassica juncea*). Cotyledon explants of an Indian gene bank accession (CR2664) and the German variety Terratop were transformed using *Agrobacterium tumefaciens* harboring binary vectors with multiple single guide RNAs to induce either large deletions or frameshift mutations in both *Bra j I* homoeologs. A total of 49 T_0 lines were obtained with up to 3.8% transformation efficiency. Four lines had large deletions of 566 up to 790 bp in the *Bra j IB* allele. Among 18 Terratop T_0 lines, nine carried indels in the targeted regions. From 16 analyzed CR2664 T_0 lines, 14 held indels and three had all four *Bra j I* alleles mutated. The majority of the CRISPR/Cas9-induced mutations were heritable to T_1 progenies. In some edited lines, seed formation and viability were reduced and seeds showed a precocious development of the embryo leading to a rupture of the testa already in the siliques. Immunoblotting using newly developed Bra j I-specific antibodies revealed the amount of Bra j I protein to be reduced or absent in seed extracts of selected lines. Removing an allergenic determinant from mustard is an important first step towards the development of safer food crops.

Keywords: mustard, Bra j I, CRISPR/Cas, transformation, food allergen, seed storage protein.

INTRODUCTION

Food allergy is considered a public health issue and the prevalence has risen in recent decades. In sensitized persons, food allergy can elicit allergic symptoms such as urticaria, itching, wheezing, dyspnea, abdominal pain, and live-threatening anaphylactic shocks (Matsuo et al., 2015). Plant food allergenic proteins are classified according to their structural or functional properties. Most of these belong to the cupin superfamily (7S and 11S seed storage proteins) or the prolamin superfamily (2S albumins, non-specific lipid transfer proteins, α-amylase/trypsin inhibitors, cereal prolamins) or are functional plant defense proteins (pathogenesis-related proteins, proteases, and protease inhibitors) (Breiteneder and Ebner, 2000; Breiteneder and Radauer, 2004). Most of the 2S albumins are heterodimeric proteins consisting of a large and a small subunit that are connected by a disulfide bond (Shewry et al., 1995).

The known allergens among the 2S albumins include Sin a I from yellow mustard seeds (*Sinapis alba*) (Menéndez-Arias et al., 1988), Bra j I from oriental or brown mustard seeds (*Brassica juncea*) (González de la Peña et al., 1991; Monsalve et al., 1993), Ber e 1 from Brazil nut (*Bertholletia excelsior*) (Nordlee et al., 1996), Jug r 1 from the English walnut (*Juglans regia*) (Teuber et al., 1998), and Ses i 2 from sesame (*Sesamum indicum*) (Beyer et al., 2002). Mustard is a significant elicitor of allergic reactions to food, with yellow and black mustard being mostly consumed in Europe, whereas the brown mustard flour (*B. juncea* L.) is most commonly used in mustard extracts in the USA and Japan (González de la Peña et al., 1991; Monsalve et al., 2001). Mustard allergy usually appears before the age of three, thus mustard is considered as a hidden allergen in the infant's diet (Rancé et al., 2000). Currently, the most effective treatment of mustard and other food

allergies is to strictly avoid ingesting allergen-containing food. However, accidental consumption of allergen-containing food is a constant issue and sensitized persons are significantly compromised in their daily life. Therefore, the development of allergen-free foods is a strategy that would make foods safe for sensitized persons and relieve them from a serious burden.

Using random mutagenesis or traditional breeding strategies to obtain allergen-free plants is difficult and time consuming, especially for polyploid crops with multiple gene copies. Genetic engineering technologies such as RNA interference (RNAi) have been used to alleviate peanut (*Arachis hypogaea*) allergy by silencing genes encoding allergens (Chandran et al., 2015; Dodo et al., 2008). Using programmable nucleases, like CRISPR/Cas9 or TALEN, targeted random mutations and deletions can be induced (Manghwar et al., 2019). This breeding method is currently the one with the greatest potential for crop improvements (Gao, 2021). Many countries have classified genome-edited crops as non-genetically modified organisms, relieving them from extensive regulation, and the first products from genome-edited plants have reached the market (Metje-Sprink et al., 2020). As a first example for a low-allergen food, CRISPR/Cas9 has been used to create low-gluten wheat (*Triticum aestivum*) for gluten-intolerant consumers (Sánchez-León et al., 2018).

Brassica juncea (L.) Czern is one of the major oilseed brassicas, and for this purpose it is mostly cultivated in semi-arid tropics of the Indian subcontinent, as well as in drier areas of Canada, Australia, China, Russia, and the northern USA (Labana and Gupta, 1993). *Brassica juncea* is a self-pollinated plant and an amphidiploid (allotetraploid) hybrid (AABB; 2n = 36), derived from the two diploid progenitors *Brassica rapa* (AA genome, 2n = 20) and *Brassica nigra* (BB genome, 2n = 16) followed by successive chromosome doubling thousands of years ago (Redden et al., 2009; Yang et al., 2016). *Brassica juncea* has been divided into four subspecies, with different morphology, quality characteristics, and usages: (i) the subspecies *B. juncea integrifolia*, used as a leafy vegetable in Asia, (ii) the subspecies *B. juncea juncea*, mostly produced for its seeds and sometimes as fodder, (iii) the subspecies *B. juncea napiformis*, mainly used as a root-tuber vegetable, and (iv) the subspecies *B. juncea taisai*, the stalks and leaves of which are used as vegetables in China (Spect and Diederichsen, 2001). The oilseed of *B. juncea* is especially rich in fatty acid and is an excellent source of natural antioxidants, but its high glucosinolate content and a fatty acid profile with a high level of erucic acid present a health issue for consumption. On the other hand, due to its high content of erucic acid with its low flash points and good combustion and lubrication qualities it can also be a valuable component in biodiesel (Premi et al., 2013). Moreover, the high glucosinolate content renders *B. juncea* an excellent product for biofumigation as a tool to alleviate the effects of soilborne pathogens and replant diseases (Hanschen and Winkelmann, 2020; Mattner et al., 2008; Yim et al., 2016).

To date, no study has reported the production of agronomically viable low-allergenic lines in *B. juncea*. However, several studies have reported the use of genetic engineering to develop varieties of *B. juncea* improved in oil and meal quality. Augustine et al. (2013) applied RNAi-based target suppression to develop low-glucosinolate *B. juncea* lines with significantly improved seed meal quality. Furthermore, Sinha et al. (2007) reported using hairpin-RNA to silence the *fatty acid elongase* gene in *B. juncea*, resulting in a decrease of erucic acid production. Yusuf and Sarin (2007) were successful in using genetic engineering methods to overexpress the γ-*TMT* gene to increase the vitamin E (α-tocopherol) level in the seeds of *B. juncea*.

To our knowledge, the use of CRISPR/Cas9 in brown mustard has not been reported yet. Here, we show that CRISPR/Cas9 can be used to precisely and efficiently mutate the *Bra j I* gene in this allotetraploid crop such that the allergenic Bra j I protein is absent from mutant seeds.

RESULTS

Design of CRISPR/Cas9 constructs with multiplex sgRNA expression cassettes targeting *Bra j I*

Two brown mustard (*B. juncea*) lines were chosen for this study, a European (Terratop) and an Indian (CR2664) one. The two *Bra j I* homoeologs in the published genome of brown mustard (*Bra j IA* and *Bra j IB*) have highly similar nucleotide sequences and are identical for both mustard lines (Figure S1). To modify the two *Bra j I* homoeologs, we designed eight single guide RNAs (sgRNAs) (sg1, sg2, sg3, sg4, sg5, sg6, sg7A, and sg7B) targeting conserved regions in the *Bra j I* exon or 5′/3′-untranslated region (UTR) regions (Figure 1a, Figure S1, and Table S1). All sgRNAs were individually driven by the *AtU6* promoter. Two final binary constructs (named pBraj1256 and pBraj3477) both carrying genes for *SpCas9*, hygromycin resistance, and sgRNA expression cassettes were generated (Figure 1b). pBraj1256 harbored four sgRNAs (sg1, sg2, sg5, and sg6) and could potentially lead to a complete deletion of *Bra j IA* and *Bra j IB* by simultaneously targeting their 5′- and 3′-UTR regions. pBraj3477 contained four sgRNAs (sg3, sg4, sg7A, and sg7B) targeting *Bra j I* coding sequences to introduce deletions or insertions at the cleavage sites that can lead to frameshift mutations of *Bra j IA* and *Bra j IB*.

Genetic transformation of mustard

Cotyledon explants of the *B. juncea* lines Terratop and CR2664 were transformed with the binary vectors containing either the reporter gene construct pEGFP (carrying an

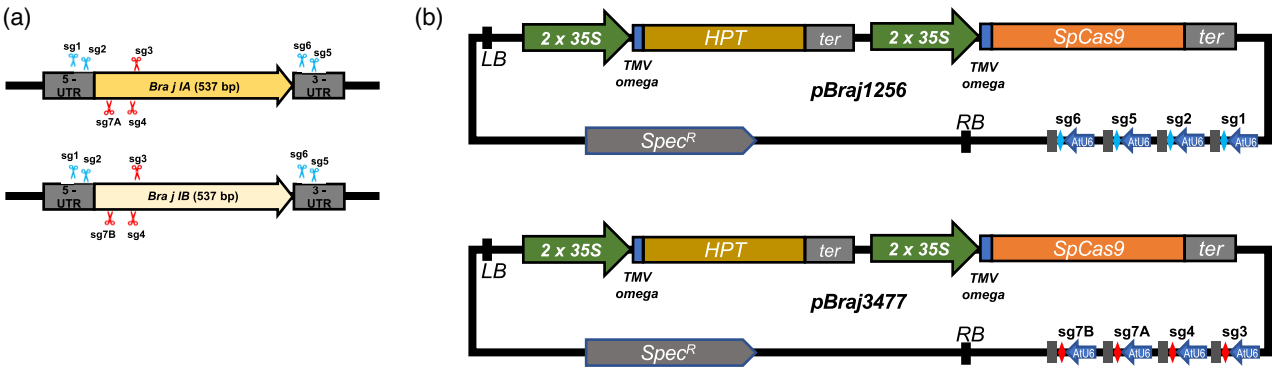

Figure 1. Constructs for targeted mutagenesis of *Bra j I* in *B. juncea* using CRISPR/Cas9. (a) Cartoon of the homoeologous *Bra j I* genes in the A and B subgenomes of tetraploid brown mustard (*B. juncea*) with sgRNA target sites (scissors). The four sgRNAs sg1, sg2, sg5, and sg6 (blue) target conserved 5′- and 3′-untranslated regions of *Bra j IA* and *Bra j IB*, whereas the four sgRNAs sg3, sg4, sg7A, and sg7B (red) target the *Bra j IA* and *Bra j IB* coding sequences. (b) Schematic of binary vectors designed for deleting (pBraj1256) or disrupting (pBraj3477) the coding sequences of *Bra j IA* and *Bra j IB*, respectively. *HPT*, *SpCas9*, and *Spec*^{R} encode hygromycin B phosphotransferase, *Streptococcus pyogenes* Cas9 endonuclease, and a spectinomycin resistance protein, respectively.

enhanced green fluorescence protein-encoding gene and a kanamycin selection marker) or one of two CRISPR/Cas9 constructs (pBraj1256 and pBraj3477, carrying a hygromycin selection marker) (Figure 2a,b). The regeneration of first putative transgenic shoots was observed 4 weeks after infection with *A. tumefaciens* (Figure 2c) and during the following three culture passages developed into plantlets (Figure 2d). Within the first 3 months, some weaker shoots died and these non-transgenic escapes were characterized by an albino-like phenotype. Only the shoots that survived after four culture passages on selection medium were considered to be transgenic shoots and were further tested by PCR. The pEGFP construct was used to establish the genetic transformation protocol (Table 1). To characterize the 57 putative transgenic plants obtained from the two CRISPR/Cas9 constructs, three vector-specific primer pairs targeting different regions of the transfer DNA (T-DNA) were used. Based on the PCR results, 19 out of 23 Terratop T_0 plants and 30 out of 34 CR2664 T_0 plants were PCR-positive plants (Figure S2). The transformation efficiency was line-dependent and also varied with the binary vectors used (Table 1) as well as among the independent experiments (Table S1). The transformation efficiencies in the line Terratop were 2 and 3.8% for pBraj1256 and pBraj3477, respectively, whereas in the line CR2664, transformation efficiencies of 3.8 and 3% were recorded with pBraj1256 and pBraj3477, respectively. In total, from the genetic transformation experiments 49 T_0 plants were transferred to the greenhouse. The surviving transgenic plants (36 T_0 plants) were grown until maturity and leaves and seeds were harvested for further characterization.

Characterization of *Bra j I* mutations in regenerated mustards

To identify CRISPR/Cas9-induced mutations in *Bra j I*, site-specific primer pairs were used to amplify the corresponding *Bra j IA* and *Bra j IB* regions from the transgenic T_0 mustard genomes. PCR on DNA from two T_0 lines (T0-1 from CR2664 and T0-47 from Terratop) which were transformed with the deletion construct pBraj1256 amplified a shorter *Bra j IB* fragment, potentially indicating a deletion (Figure S3). These DNA fragments were cloned and sequenced, revealing a 695-bp deletion of *Bra j IB* in line T0-1 (corresponding precisely to the region between the 5′- and 3′-flanking sgRNA target sites) and a 790-bp deletion of *Bra j IB* in line T0-47 (Figure S4). In addition, we found that two T_0 lines from Terratop (T0-21 and T0-55), which had been transformed with the frameshift mutation construct pBraj3477, also produced a PCR fragment indicating a deletion in *Bra j IB*. Sequencing revealed that line T0-21 has a 566-bp deletion in *Bra j IB* (the deleted region contained sg3 and sg4 target sites), and line T0-56 carries a 629-bp deletion (from the sg7 cutting site to the 3′-UTR) in *Bra j IB* (Figure S4).

Furthermore, we selected 16 transgenic T_0 lines transformed with pBraj3477 (10 from Terratop and six from CR2664) and 14 T_0 lines transformed with pBraj1256 (five from Terratop and nine from CR2664) for detailed *Bra j I* genotyping (Table 2 and Table S2). Among the 15 Terratop T_0 lines, Sanger sequencing indicated that two and five T_0 lines transformed with pBraj3477 and pBraj1256, respectively, carried indels in the targeted regions and the other eight lines did not show any mutation. For the 15 CR2664 T_0 lines, sequencing results showed that six and seven T_0 lines transformed with pBraj3477 and pBraj1256, respectively, carried indels in the targeted regions. Importantly, by specifically analyzing the *Bra j IA* and *Bra j IB* genes, we found that three CR2664 mutants (T0-22, T0-26, and T0-32) transformed with pBraj3477 had all four *Bra j I* alleles mutated (Table 2, Figure S4b).

Overall, for pBraj3477 the mutagenesis frequency of *Bra j IA*/*Bra j IB* was about 33.3 (4/12) and 100% (6/6) in

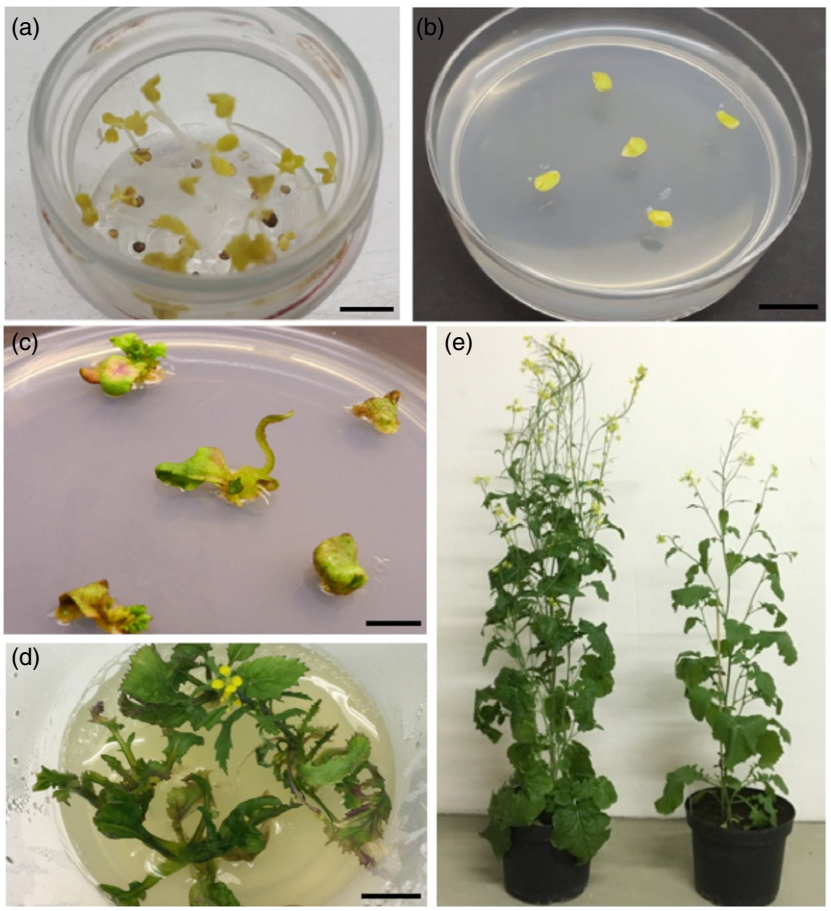

Figure 2. Generation of transgenic *B. juncea* plants (line CR2664) carrying the CRISPR/Cas9 constructs. (a) Five-day-old seedlings. (b) Cotyledon explants used for co-culture. (c) Shoot regeneration 4 weeks after co-culture with *A. tumefaciens* with pBraj1256. (d) Ten-week-old transgenic plants transformed with *A. tumefaciens* pBraj1256. (e) Flowering transgenic plants 10 weeks after acclimatization. Bars represent 1 cm.

Table 1 Transformation efficiencies for two *B. juncea* lines (Terratop and CR2664) using the three vectors pEGFP, pBraj1256, and pBraj3477

Vector[a]	Line	No. of explants[b]	No. of T_0 lines obtained	Transformation efficiency (%)[c]
pEGFP	Terratop	300	2	0.7
	CR2664	300	11	3.7
pBraj1256	Terratop	400	8	2
	CR2664	500	19	3.8
pBraj3477	Terratop	400	15	3.8
	CR2664	500	15	3

[a]Kanamycin selection for pEGFP and hygromycin selection for pBraj1256 and pBraj3477.
[b]For details see Table S1.
[c]Percentage of independent transgenic shoots per 100 explants. Transgenicity was analyzed by three different PCR reactions targeting the T-DNA.

Terratop and CR2664, respectively. Using pBraj1256, the mutagenesis frequency of *Bra j IA*/*Bra j IB* was 100 (6/6) and 80% (8/10) in Terratop and CR2664, respectively. Taken together, we successfully modified *Bra j I* in two brown mustard lines.

Inheritance of mutations in T_1 plants

To investigate the segregation of biallelic and heterozygous mutants, T_1 progenies were generated by self-pollination and analyzed. Sanger sequencing showed that 11 T_1 plants from two *bra j I* biallelic mutants (eight of them were from T0-22 and three from T0-32) were homozygous for *bra j IA* and *bra j IB* or again biallelic mutants (Table 3). For the heterozygous mutant T0-23 which harbored a biallelic mutation at the *Bra j IA* locus and a heterozygous mutation at the *Bra j IB* locus, 17 T_1 plants were analyzed, all of which were homozygous or biallelic mutations at the *Bra j IA* locus as expected. In contrast, only one offspring (line 23-7) carried a heterozygous mutation at the *Bra j IB* locus and the remaining 16 T_1 plants were homozygous wild type at the *Bra j IB* locus, which is not the expected 1:2:1 segregation ratio for a heterozygous locus after selfing (Table S3).

To assess whether the *Bra j IB* deletion identified in line T0-1 can be transmitted to the next generation, we tested 35 T_1 progeny plants, but found no PCR fragment indicating a deletion among them (Figure S5). We selected two T_1 progenies (1-1 and 1-2) from T0-1 for sequencing and new allelic mutations were detected in both *Bra j IA* and *Bra j*

Table 2 Genetic characterization of T_0 plants of the *B. juncea* lines Terratop and CR2664 regarding transgene presence and mutations in the target genes *Bra j IA* and *Bra j IB*, seed weight, and germination of seeds

Plant number	Plasmid	Line	Transgenicity assay[a]	Genotype[b] *Bra j IA* allele 1/allele 2	Genotype[b] *Bra j IB* allele 1/allele 2	Seed number obtained from free pollination	Weight of 100 seeds [g][c]	*In vitro* germination [%] (n = 10)	*Ex vitro* germination [%] (n = 20)
K2	-	Terratop	-	Wild type/wild type	Wild type/wild type	4477	0.257	100	95
K17	-	CR2664	-	Wild type/wild type	Wild type/wild type	4490	0.289	80	95
1	pBraj1256	CR2664	+	Wild type/wild type	Wild type/−695 bp	745	0.280[ns]	40	15
22	pBraj3477	CR2664	+	−14 bp (sg7)/+1 bp (sg7)	−1 bp (sg7)/+1 bp (sg7)	119	0.095	40	55
23	pBraj3477	CR2664	+	−14 bp (sg7)/+1 bp (sg7)	Wild type/+1 bp (sg7)	65	0.218	50	85
25	pBraj1256	CR2664	+	Wild type/wild type	Wild type/wild type	307	0.103***	20	20
26	pBraj3477	CR2664	+	+1 bp (sg4), +1 bp (sg7)/+1 bp (sg4), +1 bp (sg7)	+1 bp (sg7), −4 bp (sg4)/+1 bp (sg7), −1 bp (sg4)	482	0.133***	10	30
27	pBraj1256	CR2664	+	Wild type/wild type	Wild type/wild type	125	0.068	10	20
30	pBraj1256	CR2664	+	−1bp (sg1)/−1bp (sg1)	Wild type/−8 bp (sg1)	203	0.157	50	90
31	pBraj1256	Terratop	+	−1bp (sg1)/−1bp (sg1)	−4 bp (sg1)/−1 bp (sg1)	1264	0.183***	60	85
32	pBraj3477	CR2664	+	+1 bp (sg4); +1 bp (sg7)/+1 bp (sg4); +1 bp (sg7)	+1 (sg7), +1 (sg4)/−2 (sg7)	182	0.195	0	15
33	pBraj3477	CR2664	+	Wild type/−3 bp (sg7)	Wild type/+1 bp (sg7)	1303	0.198***	0	30
34	pBraj3477	CR2664	+	Wild type/−3 bp (sg7)	Wild type/−1 bp (sg7)	155	0.163	10	0
37	pBraj1256	CR2664	+	−1 bp (sg1)/−1 bp (sg1)	+1 bp (sg1)/+1 bp (sg1)	128	0.153	80	50

[a]'+' detected, '-' not detected.
[b]The sequencing chromatograms were decoded by ICE Analysis (https://ice.synthego.com/#/).
[c]For lines with less than 300 seeds no statistical analysis was conducted. For the remaining lines the 100-seed weight of three to four subsamples was compared against the wild type by Dunnett's test. ***P < 0.0001; ns, not significant.

IB alleles. T_1 line 1-1 and T_1 line 1-2 displayed homozygous 1-bp deletions (-C) at the *Bra j IA* sg1 site and a biallelic 1-bp insertion (+T/+A) at the *Bra j IB* sg1 target site (Table S3). It is possible that the T_0 plant was mosaic and the deleted allele was not present in the germline, whereas the detected new mutations occurred either in the T_0 or the T_1 generation. Nevertheless, these results show that the majority of our CRISPR/Cas9-induced mutations in mustard were heritable. We analyzed whether off-target mutations occurred during our editing approach. For this, 24 predicted off-target sites in the two edited T_1 lines 22-1 and 31-5 were PCR-amplified and analyzed by sequencing. No off-target mutation was detected (Figure S8).

Phenotyping of transgenic mustard seeds

The T_0 plants of both lines that clearly showed homozygous mutations (either monoallelic or biallelic) and the wild-type plants were analyzed for yield and viability traits (Table 2 and Table S2). Seed production was significantly reduced in some of the transgenic lines. This was especially obvious in the controlled crosses for which lower percentages of silique formation and much lower numbers of seeds per silique were recorded for most transgenic lines (Table S4). After open pollination, the wild-type plants of both lines produced approximately 4500 seeds per plant with a 100-seed weight of 0.257 g for Terratop and 0.289 g for CR2664, while only two out of 57

Table 3 Genetic characterization of T_1 plants of the *B. juncea* lines Terratop and CR2664 regarding transgene presence and mutations in the target gene *Bra j I*, seed weight, and germination of seeds

Plant number	Plasmid	Line	Transgenicity assay[a]	Genotype[b]		Seed number obtained from free pollination	Weight of 100-seed weight [g][c]	*In vitro* germination [%] (n = 10)	*Ex vitro* germination [%] (n = 20)
				Bra j I A allele 1/allele 2	*Bra j I B* allele 1/allele 2				
K17	Wild type	CR2664	-	Wild type/wild type	Wild type/wild type	3224	0.303	100	100
K2	Wild type	Terratop	-	Wild type/wild type	Wild type/wild type	2178	0.273	100	95
22-1	pBraj3477	CR2664	+	−14 bp (sg7)/ +1 bp (sg7)	−1 bp (sg7)/+1bp (sg7)	182	0.222	30	65
22-2	pBraj3477	CR2664	+	−14 bp (sg7)/ +1 bp (sg7)	+1 bp (sg7)/+1bp (sg7)	85	0.125	20	30
22-3	pBraj3477	CR2664	+	−14 bp (sg7)/ −14 bp (sg7)	−1 bp (sg7)/ −1 bp (sg7)	0	n.a.	n.a.	n.a.
22-4	pBraj3477	CR2664	+	+1 bp (sg7)/ +1 bp (sg7)	−1 bp (sg7)/+1bp (sg7)	612	0.154***	50	55
22-5	pBraj3477	CR2664	+	−14 bp (sg7)/ +1 bp (sg7)	−1 bp (sg7)/+1bp (sg7)	118	0.208	80	75
22-6	pBraj3477	CR2664	+	−14 bp (sg7)/ +1 bp (sg7)	+1 bp (sg7)/+1bp (sg7)	256	0.120	40	30
22-7	pBraj3477	CR2664	+	−14 bp (sg7)/ +1 bp (sg7)	−1 bp (sg7)/−1bp (sg7)	10	0.211	20	n.a.
22-8	pBraj3477	CR2664	+	−14 bp (sg7)/ −14 bp (sg7)	−1 bp (sg7)/+1bp (sg7)	521	0.181***	40	70
28-1	pBraj1256	CR2664	+	+1 bp (sg1)/ +1 bp (sg1)	−58 bp (sg1)/ −58 bp (sg1)	1199	0.381[ns]	100	100
31-2	pBraj1256	Terratop	-	−1 bp (sg1)/ −1 bp (sg1)	−4 bp (sg1)/ −1 bp (sg1)	1421	0.216***	100	95
31-5	pBraj1256	Terratop	+	−1 bp (sg1)/ −1 bp (sg1)	−1 bp (sg1)/ −1 bp (sg1)	3298	0.230***	80	90
32-1	pBraj3477	CR2664	+	+1 bp (sg4); +1 bp (sg7)/ +1 bp (sg4); +1 bp (sg7)	−2 bp (sg7)/ −2 bp (sg7)	1067	0.219***	40	40
32-2	pBraj3477	CR2664	+	+1 bp (sg4); +1 bp (sg7)/ +1 bp (sg4); +1 bp (sg7)	−2 bp (sg7)/ −2 bp (sg7)	700	0.430***	60	45
32-3	pBraj3477	CR2664	+	+1 bp (sg4); +1 bp (sg7)/ +1 bp (sg4); +1 bp (sg7)	+1 (sg7); +1 (sg4)/−2 bp (sg7)	1201	0.204***	70	80
35-3	pBraj1256	CR2664	+	+1 (sg1), −1 (sg 5)/+1 (sg1), −1 (sg 5)	−83 bp (sg5), −32 bp (sg2)/ −83 bp (sg5), −32 bp (sg2)	1037	0.156***	80	85
35-10	pBraj1256	CR2664	-	+1 (sg1)/+1 (sg1)	−83 bp (sg5), −32 bp (sg2)/ −83 bp (sg5), −32 bp (sg2)	47	0.095	n.a.	n.a.

[a]PCR with three T-DNA-specific primer pairs. +, fragment detected; -, no fragment detected.
[b]The sequencing chromatograms were decoded by ICE Analysis (https://ice.synthego.com/#/).
[c]For lines with less than 300 seeds no statistical analysis was conducted. For the remaining lines the 100-seed weight of three to four subsamples was compared against the wild type by Dunnett's test. ***$P < 0.0001$; ns, not significant; n.a., not analyzed.

transgenic lines produced more than 1000 seeds, i.e., lines 31 and 33 with 1264 seeds and a 100-seed weight of 0.183 g and 1303 seeds and a 100-seed weight of 0.198 g, respectively. Most of the remaining transgenic lines had a reduced seed production of less than 500 seeds per plant. The transgenic line 23 showed the lowest seed production

with only 65 seeds and a 100-seed weight of 0.218 g. A significantly reduced 100-seed weight was observed for four T_0 plants (25, 26, 31, and 33), and three further T_0 plants produced seeds of severely reduced weight (22, 27, and 36) but due to their low seed number could not be included in the statistical comparison (Table 2 and Table S2). In contrast, seeds harvested from plant 28 were significantly heavier than those of the wild type (Table S2). The seeds from the T_0 plants were germinated *in vitro* and *ex vitro* to evaluate seed viability. *In vitro*, the T_0 lines 35 and 37 showed the highest germination rates of 100% and 80%, respectively, whereas the seeds of T_0 lines 29, 32, and 33 did not germinate at all. *Ex vitro*, the highest germination rate of 90% was observed with line 30, followed by lines 23 and 31, with a germination rate of 85%. No germination was recorded for seeds of lines 24, 29, and 34.

The seeds from those T_1 lines that carried CRISPR-induced homozygous mutations were harvested and the 100-seed weight was as well determined (Table 3). Seed production was again much lower in most T_1 lines compared to wild-type plants, and the seed weight was reduced and strongly varied from plant to plant. Seed production of the T_1 plants of line 22 ranged from 0 to 612 seeds with a 100-seed weight of 0.120 g to 0.208 g. However, the T_1 plant 31-5 of line 31 showed a production of 3298 seeds, which was even higher than that from the wild type. When testing the germination ability, the *in vitro* germination rate of the seeds of the T_1 plants of line 22 ranged from 20 to 80%, and *ex vitro* germination was comparable (from 30 to 75%). For all remaining T_1 plants, the *in vitro* germination rate varied from 30 to 100% and the *ex vitro* germination rate varied from 40 to 100%, with most T_1 lines germinating at lower percentages than wild type.

In some transgenic lines, the seed phenotype clearly differed from that of wild-type plants (Figure 3). Some T_0 and

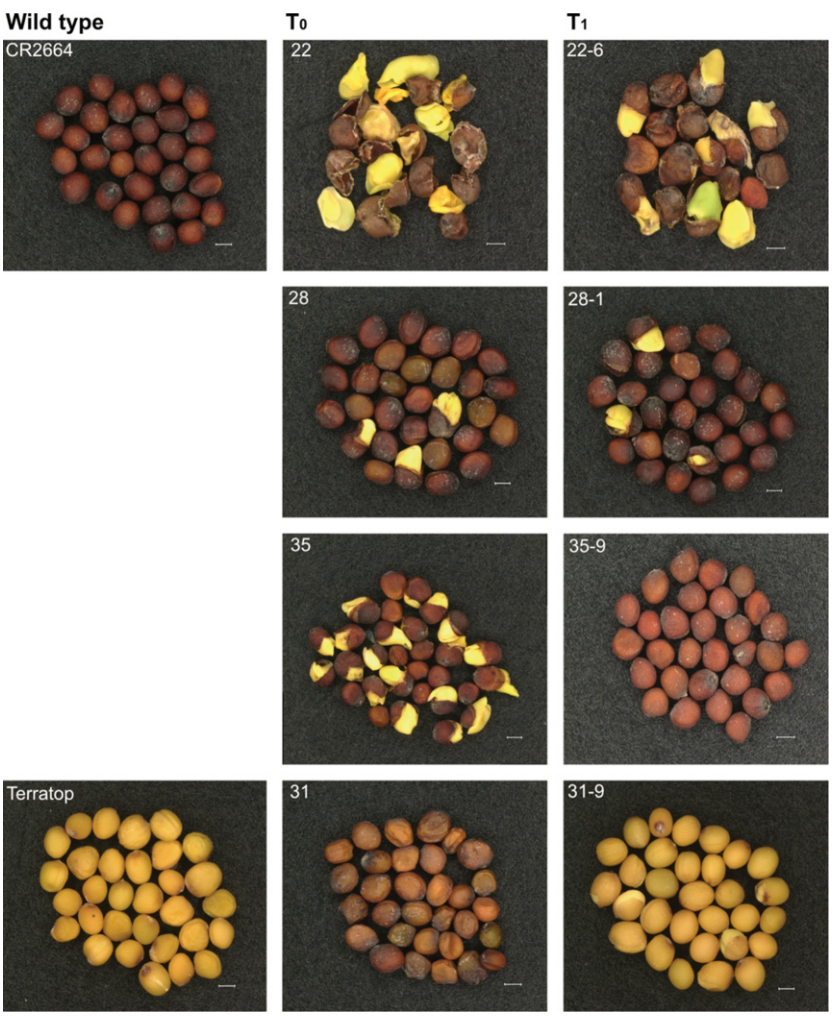

Figure 3. Seeds from wild-type, T_0, and T_1 lines of *B. juncea* (line CR2664 and Terratop) carrying the CRISPR/Cas9 constructs. Numbers 22, 28, 35, and 31 refer to T_0 lines (see Table 2 and Table S2); 22-6, 28-1, 35-9, and 31-9 refer to T_1 lines (see Table 3 and Table S3). Bar = 500 μm.

T_1 seeds showed a precocious development of the embryo leading to a rupture of the testa and a breakthrough of the embryo through the seed coat already in the siliques. T_0 and T_1 plants of line 22 were heavily affected with more than 70% of the seeds showing this abnormality. The T_0 and T_1 plants of line 28 only rarely expressed this phenotype, whereas in line 35 the T_0 was heavily affected while the seeds of the T_1 plant lines were less affected. For the T_0 line 31 of cultivar Terratop, a change in the seed coat color was observed from yellow to brown, which reverted to yellow in some of the T_1 lines (line 31-9) (Figure 3).

Immunodetection of Bra j I in T_1 mustard seeds

The seed storage protein Bra j I is a 2S albumin with a molecular mass of about 22 kDa which is processed into two subunits of 9.5 kDa and 12 kDa (Breiteneder and Ebner, 2000; L'Hocine et al., 2019). Two bands of corresponding sizes are visible in Coomassie-stained protein profiles of seed extracts from wild-type and EGFP-transgenic plants (Figure 4a–d). These protein bands are not visible in seed extracts from T_1 lines with frameshift mutations in all *bra j I* alleles (different T1-22 lines; Figure 4a), indicating that the induced mutations successfully block Bra j I protein production. In contrast, the Bra j I protein bands are reduced in their abundance but not completely absent in the T_1 lines T1-32-1 and T1-35-3 (Figure 4b,d). Line T1-32-1 carries frameshift mutations in both *Bra j IA* and *Bra j IB*, and line T1-35-3 carries mutations in the 5′- and 3′-UTRs of *Bra j I*, but not within the coding region (Table 3).

To analyze whether the identified protein bands correspond to the Bra j I protein, two specific antibodies (KRO58-A3 and STE2-G2) were generated by antibody phage display and used in immunoblotting with seed extracts (Figure 4e–h). Both antibodies were selected against the linear immunogenic epitope of Bra j I (Figure S6) (Monsalve et al., 1993). The antibodies were cloned into the scFv-Fc format (with the human IgG1 Fc fragment) and produced in EXPI293F cells. The binding of both antibodies to complete Bra j I was validated by ELISA on recombinant Bra j I. To determine the EC_{50} values, a titration ELISA was performed (Figure S7), resulting in subnanomolar EC_{50} values for both antibodies: 0.22 nM for KRO58-A3 and 0.32 nM for STE2-G2 (KRO58-A3 was chosen for the immunoblotting). As expected, the anti-Bra j I antibody efficiently detected a protein in wild-type and EGFP-transgenic seed extracts corresponding to the heavy chain (12 kDa) of Bra j I (Figure 4e–h). No anti-Bra j I antibody binding was detected in lines T1-22 and T1-32-1, verifying that the Bra j I protein was absent in seeds of these T_1 *bra j I* mutants (Figure 4e,f).

To further estimate whether mutation of *Bra j IA*, but not *Bra j IB* results in reduced Bra j I protein accumulation within the seeds, we selected four T_1 *bra j I* mutants from T_0 line 23: T1-23-8, T1-23-12, T1-23-15, and T1-23-16, with biallelic or homozygous mutations at *Bra j IA* but no editing at *Bra j IB* (Table S3). Surprisingly, practically no Bra j I protein accumulated in these four T1-23 lines (Figure 4c,g), suggesting that the intact *Bra j IB* alleles in these T_1 lines did not result in a partial protein accumulation.

In contrast, the T_1 lines T1-31-5 and T1-35-3 showed a reduced accumulation of Bra j I protein. Lines 31-5 and 35-3 contain mutations at the 5′- and/or 3′-UTR regions of *Bra j IA* and *Bra j IB*, but not the coding regions (Table 3). This suggests that mutations in these regions might also have an impact on the level of Bra j I production. Taken together, we successfully demonstrated that several of our edited mustard lines have no or a reduced accumulation of Bra j I protein.

DISCUSSION

Transformation efficiency differs between genotypes

The genetic transformation of both mustard lines (Terratop and CR2664) using CRISPR/Cas9 editing technology was successfully achieved. In this study, we focused on the two mustard lines CR2664, originating from India with a brown testa, and Terratop, derived from a German breeder and selected for being used as a catch crop with a yellow testa. The Indian *B. juncea* lines contain higher levels of erucic acid and butenyl and propenyl glucosinolates, while the European lines were selected for a low erucic acid level and only contain propenyl glucosinolates (Lionneton et al., 2004; Vaughan et al., 1963). Fazekas et al. (1986) reported good amenability to tissue culture of the Indian lines, with greater potential for adventitious shoot regeneration than the European lines. Since the regeneration capacity is the precondition for *A. tumefaciens*-mediated transformation, most of the transformation experiments in *B. juncea* reported so far involved Indian lines (Ahmed et al., 2017; Bhuiyan et al., 2011; Dutta et al., 2008; Mondal et al., 2007; Rani et al., 2017; Sharma et al., 2004; Singh et al., 2009; Thakur et al., 2020). This paper is one of the first reporting successful *A. tumefaciens*-mediated transformation of a European line of *B. juncea*. The transformation efficiencies obtained in this study from both lines (Terratop and CR2664) varied between 0.7 and 3.8%, thus being similar to the efficiency of 0.5 to 1.5% reported by Mondal et al. (2007) but lower than the previously reported transformation efficiencies of 6–7 (Sharma et al., 2004), 16.2 (Singh et al., 2009), or 16.4 to 19.7% (Rani et al., 2017). Factors that could improve the transformation efficiencies in future experiments could involve a delayed start of selection for 3–5 days (as shown by Bhuiyan et al., 2011) or a preculture of 2 days as suggested by Thakur et al. (2020).

Successful genome editing in polyploid species

Polyploidization in flowering plants has promoted genetic variation by genome duplication during their adaptive

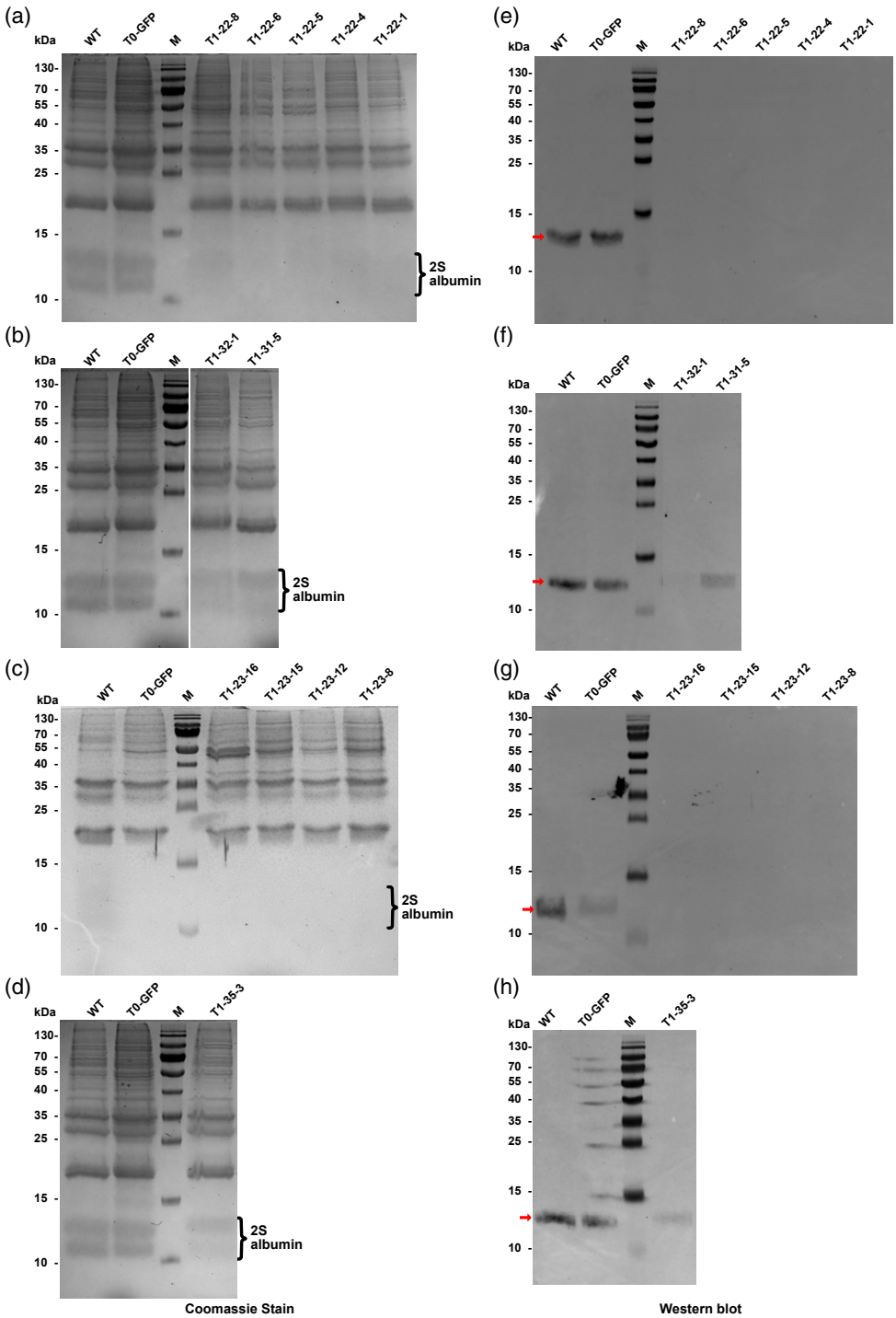

Figure 4. Loss of Bra j I protein in genome-edited mustard seeds. (a–d) Electrophoretic profiles of seed proteins from T_1 plants. The concentrations of protein extracts were normalized and about 40 μg was loaded per lane. The proteins were separated by SDS-PAGE and stained with Coomassie Blue R-250. (e–h) Immunoblot analysis of Bra j I protein in seeds from T_1 mustard plants. Following SDS-PAGE, proteins were transferred to a nitrocellulose membrane. The Bra j I -specific monoclonal antibody KRO58-A3 was used for Bra j I (red arrow, heavy chain, approximately 12 kDa) detection. WT, wild-type CR2664 seeds; T0-GFP, EGFP-transgenic seeds (non-edited control); M, protein marker.

evolution. A large number of crops are alloploids (e.g., rapeseed [*B. napus*], wheat, and cotton [*Gossypium hirsutum*]) combining genomes from different species (Pelé et al., 2018). Random mutagenesis technologies to improve traits in polyploid crops are usually inefficient; however, targeted genome editing has become a promising

alternative. The CRISPR/Cas9 system is the most widely used genome editing technology for precise plant genome modifications (Chen et al., 2019). Until now, CRISPR/Cas9-mediated genome editing has been successfully reported in some polyploid crops: In allotetraploid crops, the editing efficiencies varied; for example, the mutagenesis efficiency ranged from 96.8 to 100% in oilseed rape (*B. napus*) (Li et al., 2018) and from 66.7 to 100% in cotton (*G. hirsutum*) (Wang et al., 2017). A previous report showed that the Arabidopsis U6 small nuclear RNA (snRNA) promoter *AtU6-26* has a higher transcriptional activity than other *AtU6* snRNA promoters (Li et al., 2007), and in allotetraploid sweet basil (*Ocimum basilicum*) the mutation efficiency was 92.6% by using such an *AtU6-26*-sgRNA expression cassette (Navet and Tian, 2020). In this study, we utilized a multiplex CRISPR/Cas9 system (harboring the *AtU6-26*-sgRNA expression cassettes) to simultaneously edit four alleles of *Bra j I* in mustard, and the mutation efficiencies for *Bra j IA* and *Bra j IB* were 47.1 (8/17) and 50% (9/18) in Terratop and 81.3 (13/16) and 87.5% (14/16) in CR2664. These data implied that CRISPR/Cas9 is a suitable approach and our results confirmed that high editing efficiencies can be obtained also in mustard using a *35S* and *AtU6-26* promoter for *SpCas9* and sgRNA expression, respectively.

Off-target mutations are a concern for the applied use of CRISPR/Cas9. Such off-target events can be limited by using one of the high-fidelity SpCas9 variants instead of the wild-type SpCas9 (Zhang et al., 2017) or by choosing sgRNAs with few predicted off-targets (Liu et al., 2017). In plant research, possible off-target events can sometimes be removed by crossing or segregation in the next generation while keeping the desired mutations. In this study, we analyzed 24 predicted off-target sites in two edited T_1 lines and did not find any off-target mutation. Because these lines still carry the CRISPR/Cas9 transgene, it is possible that mutations will occur at a later time point or at a locus that was not analyzed. Before commercial application, a more thorough analysis might be advisable.

Altered seed phenotype in some transgenic lines

We noted that the seeds of several *bra j I* mutants were aberrant in shape (Figure 3), weight, and germination efficiency (Table 2, Table 3, Table S2, and Table S3). Those phenotypes indicate that Bra j I may have an influence on seed development. The seed yield and related traits revealed wide phenotypic variation between the wild-type plants and the transgenic T_0 and T_1. In this study, the wild types of both lines produced approximately 4500 seeds per plants with a 100-seed weight of 0.257 g for Terratop and 0.289 g for CR2664 (Table 2). The number of seeds is similar to the numbers reported by Stevens (1932, 1957) and the weight is within the range of 2.63 g and 1.84 g (1000-seed weight) reported by Stevens (1932, 1957), whereas the seed weight was higher than that recorded by Yoshimura et al. (2016) (1000-seed weight: 1.57 g). In contrast, Ramana and Ghildiyal (1997) reported much higher seed production of 3825 to 7075 per plant with a 1000-seed weight ranging from 4.49 to 4.64 g. In this study, a much lower seed production was observed for some of the transgenic lines, and this reduction was also found for the 100-seed weight and seed viability. The lower seed number might be caused by the isolation bags that had to be used for the transgenic plants as soon as flowers started to open. However, the significant reduction of seed weight and viability could also be the result of modified Bra j I levels in edited *B. juncea* seeds. Rolletschek et al. (2020) reported a seed weight reduction in transgenic lines when using RNAi to suppress seed storage protein synthesis in *B. napus*. Moreover, they observed an irregular shape of the cotyledons of mature transgenic seeds with a volume of void spaces significantly greater than in the wild-type seeds and the transgenic embryos being 7 to 9% smaller than the wild-type ones. For T_0 line 25, we could not detect a mutation in the *Bra j I* homoeologs, but observed a significantly reduced seed weight. Since this plant was proven to contain the editing construct, this fact could be explained by chimerism and a random selection of a leaf that did not carry mutations. A negative effect due to the integration site of the transgene could be an alternative explanation.

The allergen Bra j I belongs to the 2S albumin class, one of the most abundant seed storage protein classes in mustard (Monsalve et al., 1993). The seed storage proteins are essential for the seed germination vigor and provide an efficient scavenging system of the reactive oxygen species (ROS) actively generated during seed germination and, therefore, protect the other proteins that are essential for germination from oxidation (El-Maarouf-Bouteau et al., 2013; Muntz et al., 2001; Nguyen et al., 2015). Some of our T_0 and T_1 seeds showed a precocious development of the embryo leading to a rupture of the testa already in the siliques (Figure 3). Similar abnormalities were reported in F_1 hybrid seeds derived from a cross of transgenic *B. napus* and *B. juncea* that showed cracked seed coats (Tang et al., 2018). The phenotype is similar to that of mutants lacking a growth arrest during seed development and being deficient in dormancy resulting in vivipary. Vivipary occurs in mutants lacking genes that suppress germination and here especially adequate abscisic acid (ABA) levels are important to prevent sprouting (Bewley et al., 2012). The level of dormancy in a seed, i.e., its capacity to repress precocious germination, is mostly related to the capacity of the seed to synthesize ABA in the endosperm and in the embryo, which is an essential contributor to maintain dormancy (Lee et al., 2010). The reduction in seed storage protein levels in seed development often results in a low dormancy level of the seeds (Debeaujon et al., 2000; Nguyen et al., 2015; Sugliani et al., 2009). A possible link to ABA

can be the levels of free amino acids, which can be assumed to be altered in the *Bra j I* mutant lines. Scuffi et al. (2014) were able to show that hydrogen sulfide acts as a component of the ABA signaling pathway that is involved in stomatal closure. Hydrogen sulfide can be enzymatically produced from cysteine via L-cysteine desulfhydrase (Scuffi et al., 2014).

Mutations led to the intended decreased Bra j I content in seeds

The allergen Bra j I from brown mustard was initially found in the 2S albumin fraction and recognized by the IgE of sensitive individuals (L'Hocine et al., 2019). To create Bra j I-free mustard, the *Bra j I* gene was inactivated in the genome using CRISPR/Cas9. Three regenerated T_0 *bra j I* mutant lines (lines 22, 26, and 32) were identified to contain frameshift mutations in all four *Bra j I* alleles. The corresponding seeds of T_1 offsprings of T_0 lines 22 and 32 showed significantly less 2S albumin compared to wild-type seeds. Moreover, the immunoblotting results with an anti-Bra j I antibody showed no Bra j I protein in all six of these lines, confirming that we successfully obtained Bra j I-free mustard lines. These lines are a valuable start to develop low-allergenic mustard. We noticed increased abundance of some proteins in the seed extracts of T_1 lines, e.g., T1-23-16 or T1-22-5 (Figure 4a,c), possibly indicating that the loss of Bra j I caused an accumulation of other seed storage proteins. This is in agreement with recent findings in *Camelina sativa* cruciferin C knockout lines (Lyzenga et al., 2019).

CRISPR/Cas9-induced DNA double-strand breaks (DSBs) are mainly repaired by non-homologous end joining (NHEJ) and usually result in small insertions or deletions (indels). Indels can alter the gene-coding frame and create a premature stop codon. However, indels also produce pseudo-mRNAs that do not encode functional proteins and lead to unexpected effects in CRISRP-based gene knockout studies (Tuladhar et al., 2019). Besides, such pseudo-mRNAs from the truncated reading frame of a targeted gene could produce proteins with altered amino acid sequences. Thus, a clean genetic mutation would require to completely delete a gene using two or more sgRNAs simultaneously. In our study, we expressed four sgRNAs in tandem to delete the whole *Bra j I* coding region, and Sanger sequencing confirmed that the *Bra j IB* allele was deleted by the joining of the sg1 and sg5 cleavage sites in the T_0 plant line 1 (Figure S4). However, in another line, line 47, the *Bra j IB* deletion was an outcome of an NHEJ repair with DSBs occurring in sg1 or sg2 targeted sites (Figure S4). Similar outcomes were also found in line 21 and line 56, which have large fragment deletions at cleavage sites (Figure S4). We noticed that the efficiencies of complete deletions of *Bra j IA* and *Bra j IB* were lower than we expected, and neither a deletion of *Bra j IA* nor a complete *Bra j IB* deletion line was obtained. Several studies already showed that utilizing microhomology-mediated end joining (MMEJ)-assisted CRISPR/Cas9 editing, the deletion efficiency can be increased (Owens et al., 2019; Tan et al., 2020). An alternative approach might thus be to design sgRNAs targeting microhomologous sequences to induce a complete deletion of *Bra j IA* and *Bra j IB* by the MMEJ DNA repair mechanism.

CONCLUSIONS AND OUTLOOK

The Bra j I mutation lines that were generated in this work now serve as a key starting point to further study the impact of changes in seed storage protein composition in mustard. Future work will correlate specific changes in seeds with their allergenic potential for patients and their food quality. In principle, it might even be possible to precisely change epitopes in proteins that trigger IgE binding using precision genome editing (base editors or prime editors; Anzalone et al., 2020) without removing the protein altogether and thereby avoiding altered seed phenotypes.

The protocols established for genetic transformation and CRISPR/Cas9-mediated genome editing in *B. juncea* pave the way for application in modern breeding programs of this allotetraploid crop. Compared to traditional breeding, genome editing-based new breeding technologies accelerate crop improvement since they can generate precise genetic changes and desired traits in complex genetic backgrounds in a relatively short time (Gao, 2021). Our work demonstrates the removal of an allergenic protein from brown mustard potentially improving the safety of mustard-derived food products for sensitized individuals. This work also highlights the possibility for creating hypoallergenic food plants in general to enhance their safety for human consumption.

EXPERIMENTAL PROCEDURES

Plant material and explant preparation

Seeds of two brown mustard (*B. juncea* L.) lines were used, the European cultivar Terratop (kindly provided by the breeder P.H. Petersen Saatzucht Lundsgaard GmbH, Grundhof, Germany) and an Indian line of *B. juncea* (L.) Czern. with accession number CR2664 obtained from the gene bank of the IPK Gatersleben, Germany. The mature seeds of both lines were surface-disinfected with 1% NaOCl with 0.1% Tween 20 for 5 min followed by three washing steps with sterile deionized water under aseptic conditions. The treated seeds were germinated on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) for 5 days in darkness at 24°C. The 5-day-old seedlings were collected under aseptic conditions, and the cotyledons were vertically divided into two halves and used as explants for the genetic transformation experiments.

Vector constructs

All the vectors used in this work were assembled based on Modular Cloning-compatible vectors (Table S5) (Weber et al., 2011). In

short, to construct pEGFP, 2x*35S*::*nptII* and 2x*35S*::*EGFP* transcriptional units were ligated at positions 1 and 2, respectively, into pAGM8031 by Golden Gate assembly. Suitable target sequences of sgRNAs (Table S6) were chosen using the online web tool CRISPR-P (Liu et al., 2017). Complementary oligos with target sequences were synthesized by Microsynth AG (Balgach, Switzerland). Oligonucleotide pairs were annealed and cloned into an intermediate vector (pDI1, pDI2, pDI3, and pDI4E) with *Bpi*I, yielding *AtU6-26*::*sgRNA* expression cassettes. The 2x*35S*::*HPT* transcriptional unit and the 2x*35S*::*SpCas9* transcriptional unit together with four sgRNA (sg1, sg2, sg5, sg6) expression cassettes were ligated into pAGM8031 by Golden Gate assembly, yielding the final construct pBraj1256. The 2x*35S*::*HPT* transcriptional unit and the 2x*35S*::*SpCas9* transcriptional unit and four sgRNA (sg3, sg4, sg7A, sg7B) expression cassettes were ligated into pAGM8031 by Golden Gate assembly, resulting in the final construct pBraj3477. The final constructs were transformed into *A. tumefaciens* strain LBA4404 for transformation of plants. All oligonucleotides used in this work are listed in Table S7.

Transformation of mustard plants

Glycerol stocks of the *A. tumefaciens* strain LBA4404 harboring the binary vectors pEGFP, pBrj1256, and pBrj3477 were plated on YEB medium (0.5% [w/v] sucrose, 0.5% [w/v] tryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] beef extract, 0.05% [w/v] magnesium sulfate, 1.4% [w/v] micro agar, pH 7.2) supplemented with 100 mg L^{-1} rifampicin and 100 mg L^{-1} spectinomycin and incubated for 24 h at 28°C. The bacteria were then collected from the plates and dissolved in simplified induction medium (Alt-Moerbe et al., 1988) containing 20 mM sodium citrate and 2% sucrose (pH 5.2) supplemented with 100 μM acetosyringone and 1 mM betaine hydrochloride (James et al., 1993) and incubated at room temperature for 4 h until an OD_{600} of 1.8 to 2.0 in the 1:10 dilution was reached. Medium without bacteria was used for control variants. The cotyledon explants were immersed in the different solutions supplemented with 0.03% Silwet L-77 for 10 min, blotted dry on sterile paper towel, and co-cultivated on shoot induction medium (full-strength MS medium supplemented with 3% [w/v] sucrose, 0.8% [w/v] agar, 8.88 μM benzyladenine, 5.37 μM naphthalene acetic acid, and 100 μM acetosyringone) for 3 days. After co-cultivation, the explants were washed in sterile deionized water and placed onto regeneration medium (shoot induction medium + 10 μM $AgNO_3$) and the different selection media depending on the construct (shoot induction medium + 10 μM $AgNO_3$ + 15 mg L^{-1} hygromycin for Terratop or 20 mg L^{-1} hygromycin for CR2664 with the CRISPR/Cas9 constructs and 50 mg L^{-1} kanamycin for both lines with the EGFP construct) for shoot organogenesis at 24 ± 1°C with a 16/8 h day/night cycle (35–40 μmol m^{-2} sec^{-1}, fluorescent tubes). After 28 days, the obtained shoots were separated and cultivated on the same media without $AgNO_3$ for 28 days. Surviving shoots were subcultured every 4 weeks for another 56 days. Four months after co-culture, putative transformants (T_0) were obtained. The plants from the non-transformed control (wild type) and the T_0 transgenic plants were acclimatized and grown in 5-L pots containing growing medium (3:1 mixture of peat and lime-free clay and fertilized with 1 ‰ Ferty3 Mega produced by Einheitserdewerke Patzer, Patzer Erden GmbH, Germany) under a 16/8 h day/night cycle using a Philips SON-T Agro 400 W at 22°C in the greenhouse for 4 months. Before anthesis of the first flowers, the plants were covered with isolation bags (Crispac-Beutel SM 330 × 750 mm, Baumann Saatzuchtbedarf GmbH, Germany) and shaken from time to time to improve self-pollination. Where possible, 20 flowers per plants were emasculated before anthesis and the anthers with mature pollen grains were collected from other flowers of the same plant for pollination (Table S4). At maturity, seeds were harvested to obtain the seeds for the T_1 generation.

Seed germination

The germination rates of seeds from wild-type, T_0, and T_1 transgenic plants were determined *in vitro* and in the greenhouse. For *in vitro* germination, 10 seeds from each plant were randomly chosen, subjected to surface disinfection, and incubated in darkness for 7 days at 24°C. A seed was scored as germinated 7 days after sowing when the radicle and the hypocotyl had emerged. For *ex vitro* germination, 20 seeds from wild-type, T_0, and T_1 transgenic plants were directly sown into multipot trays or 8-cm pots containing growing medium (3:1 mixture of peat and lime-free clay and fertilized with 1 ‰ Ferty3 Mega produced by Einheitserdewerke Patzer, Patzer Erden GmbH, Germany) under a 16/8 h day-night cycle using a Philips SON-T Agro 400 W, at 22°C in the greenhouse. The germination was scored after 21 days when the cotyledons had emerged above the substrate.

Seed characterization

The harvested seeds from each plant were counted with a counting machine (Contador, Pfeuffer, Kitzingen, Germany) to quantify the seed set per plant. Where possible, four times 100 seeds from each individual plant were randomly selected and the weight was determined using a precision balance (Denver Instrument SI-203.1, Cole-Parmer GmbH, Germany). Later, 15 to 30 seeds were randomly selected and photographed with a digital microscope (Figure 3) (VHX-S750E, Keyence, Japan).

Statistical analysis was conducted on the 100-seed weight using R (R Core Team, 2021) with the help of the packages tidyverse (Wickham et al., 2019) and dplyr (Hadley et al., 2021). The R package emmeans was also used to conduct pairwise comparisons (Dunnett test) (Lenth, 2021).

Identification of mutants

Frozen leaf samples were disrupted with a TissueLyser (QIAGEN GmbH, Germany) and genomic DNA was extracted using the innuPREP Plant DNA Kit (Analytik Jena, Germany) according to the instructions of the manufacturer. Three pairs of primers (hyg-F/hyg-R, F1/R1, and F2/R2) were used to detect transgenic mustards with T-DNA integrated into the genome. Subsequently, *Bra j IA* and *Bra j IB* were amplified by Q5 High-Fidelity Polymerase (NEB) from genomic DNA with site-specific primer pairs BrajA-F/BrajA-R and BrajB-F/BrajB-R (Table S7), respectively. The PCR amplicons were purified from the agarose gels using the GeneJET Gel Extraction Kit (Thermo Scientific Fermentas, USA) and sequenced (Microsynth Seqlab, Göttingen, Germany) or subcloned into pUC57 vector before sequencing. The Sanger sequencing chromatograms were decoded manually and using the ICE Analysis tool from SYNTHEGO (https://ice.synthego.com).

Generation of a monoclonal Bra j I-specific antibody

Antibody selection was performed as described previously (Russo et al., 2018) with modifications. In brief, antibodies were selected by phage display using the naïve antibody gene libraries HAL9 and HAL10 (Kügler et al., 2015) on a biotinylated peptide (Monsalve et al., 1993) (Peps4LS GmbH, Heidelberg, Germany) in microtiter plates (Corning, New York, USA). Selected antibodies were cloned into scFv-Fc format and were produced in EXPI293F cells as previously described (Wenzel et al., 2020). Titration ELISA

was performed with the selected antibodies. For a more detailed description see Supplementary Material.

Immunoblotting

Seeds were ground into a fine powder with 25% (w/v) extraction buffer (0.2 M Tris-Cl, pH 8.0, containing 0.1 M NaCl, 10 mM EDTA, 0.1% $MgCl_2$, 10% glycerol, 1 mM PMSF, and 10 nM β-mercaptoethanol) and centrifuged at 14 000 ***g*** for 10 min at 4°C. The supernatants were transferred to new tubes and centrifuged again at 11 304 ***g*** for 10 min at 4°C. These supernatants were used for further analysis. Total protein concentrations were measured using Bradford reagent (Carl Roth GmbH, Karlsruhe, Germany). Samples were boiled with loading dye at 95°C for 10 min. Protein samples were separated by 15% SDS-PAGE and visualized by staining with Coomassie Brilliant Blue R-250. For Bra j I detection, proteins were transferred onto 0.45 μm nitrocellulose membranes using a wet blotting system. Membranes were blocked overnight at 4°C in blocking buffer (TBST buffer with 5% defatted milk and 3% bovine serum albumin). Subsequently, membranes were incubated with 100 ng ml^{-1} primary antibody for 3 to 4 h at room temperature. After washing the membranes three times for 5 min each in TBST buffer, they were incubated with 1:20 000 diluted alkaline phosphatase-conjugated goat anti-human IgG secondary antibody (Dianova 109-055-98) for 1 h at room temperature. Then, the membranes were washed three times with TBST buffer for 5 min each and equilibrated with substrate buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for 10 min. Thereafter, the membranes were stained with NBT/BCIP substrate staining solution and the staining reaction was stopped by washing three times with H_2O after the signal developed. Images were taken by a ChemiDoc™ Touch Imaging System (Bio-Rad).

Off-target detection

Off-target sites were predicted by the online tool CRISPR-P (Liu et al., 2017). Based on the off-target score, the top three predicted off-target sites for each of the eight used sgRNAs were selected as potential off-target sites (Figure S8). Those sites were amplified from the genome of two selected T_1 plants by PCR and the PCR product was sequenced. Primer pairs for off-target amplification are added to Table S7.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

TW, JB, and MH conceived the study and its design, coordinated the research together with TR, and assisted with interpretations of results. JA, DZ, KR, and SS designed and performed the experiments and analyzed the data. JA, DZ, TW, JB, and KR wrote the manuscript. TR provided the recombinant Bra j I protein.

DATA AVAILABILITY STATEMENT

All relevant data can be found within the article and supporting materials.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Sequence alignment of *Bra j I* homoeologs in *B. juncea* varieties Terratop and CR2664.

Figure S2. Outcomes of the PCR test for transgenicity of all obtained mustard lines.

Figure S3. PCR amplification of both *Bra j I* homoeologs from genomic DNA of the T_0 lines.

Figure S4. Analysis of deletions in *Bra j I* in mutated T_0 lines.

Figure S5. Analysis of the *Bra j I* homoeologs of T_1 offsprings to screen for inheritance of the deletion from the T0-1 line.

Figure S6. Amino acid sequence alignment of Bra j I homoeologs in *B. juncea* varieties Terratop and CR2664.

Figure S7. Titration-ELISA for EC_{50} determination on Bra j I peptide.

Figure S8. Off-target detection.

Table S1. Transformation efficiencies for two *B. juncea* lines (Terratop and CR2664) in different experiments using the three vectors pEGFP, pBraj1256, and pBraj3477.

Table S2. Genetic characterization of T_0 plants regarding transgene presence, seed weight, and germination of seeds of the *B. juncea* lines Terratop and CR2664.

Table S3. Genetic characterization of T_1 plants regarding transgene presence, seed weight, and germination of seeds of the *B. juncea* lines Terratop and CR2664.

Table S4. Controlled pollinated flowers of T_0 plants of *B. juncea* lines Terratop and CR2664 with the number of seeds and seed weight.

Table S5. MoClo-compatible vectors used in this study.

Table S6. sgRNA sequences.

Table S7. Oligonucleotides used in this study.

Supplementary Material. Antibody selection in microtiter plate.

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