Plant Biotechnology Journal Open Access

Plant Biotechnology Journal (2023) **21**, pp. 2182–2195 doi: 10.1111/pbi.14121

Targeted editing of multiple homologues of *GTR1* and *GTR2* genes provides the ideal low-seed, high-leaf glucosinolate oilseed mustard with uncompromised defence and yield

Avni Mann[1,†], Juhi Kumari[1,†], Roshan Kumar[1], Pawan Kumar[1], Akshay K. Pradhan[2], Deepak Pental[2] and Naveen C. Bisht[1,*]

[1]*National Institute of Plant Genome Research, New Delhi, India*
[2]*CGMCP, University of Delhi South Campus, New Delhi, India*

Received 26 October 2022;
revised 21 June 2023;
accepted 26 June 2023.
**Correspondence* (Tel +91 11 26735183; fax +91 11 26741658; email ncbisht@nipgr.ac.in)
[†]These authors contributed equally.

Keywords: Brassica oilseeds, Canola quality, CRISPR/Cas9, gene editing, glucosinolate transporters, plant defence.

Summary

Glucosinolate content in the two major oilseed *Brassica* crops—rapeseed and mustard has been reduced to the globally accepted Canola quality level (<30 μmoles/g of seed dry weight, DW), making the protein-rich seed meal useful as animal feed. However, the overall lower glucosinolate content in seeds as well as in the other parts of such plants renders them vulnerable to biotic challenges. We report CRISPR/Cas9-based editing of glucosinolate transporter (*GTR*) family genes in mustard (*Brassica juncea*) to develop ideal lines with the desired low seed glucosinolate content (SGC) while maintaining high glucosinolate levels in the other plant parts for uncompromised plant defence. Use of three gRNAs provided highly efficient and precise editing of four *BjuGTR1* and six *BjuGTR2* homologues leading to a reduction of SGC from 146.09 μmoles/g DW to as low as 6.21 μmoles/g DW. Detailed analysis of the *GTR*-edited lines showed higher accumulation and distributional changes of glucosinolates in the foliar parts. However, the changes did not affect the plant defence and yield parameters. When tested against the pathogen *Sclerotinia sclerotiorum* and generalist pest *Spodoptera litura*, the *GTR*-edited lines displayed a defence response at par or better than that of the wild-type line. The *GTR*-edited lines were equivalent to the wild-type line for various seed yield and seed quality traits. Our results demonstrate that simultaneous editing of multiple *GTR1* and *GTR2* homologues in mustard can provide the desired low-seed, high-leaf glucosinolate lines with an uncompromised defence and yield.

Introduction

The allopolyploid *Brassica* species, *Brassica napus* (commonly referred to as rapeseed) and *B. juncea* (referred to as mustard) have emerged as significant oilseed crops due to increasing demand for edible oils globally. The seed meal left after oil extraction is rich in proteins and a promising substitute for soybean-based protein feed for livestock (Gatrell *et al.*, 2014). However, the presence of a high amount of glucosinolates in the seeds, and in addition, their hydrolysis products in the seed meal reduces the value of the meal as it is unpalatable and harmful, particularly to swine and poultry (Bell *et al.*, 2018; Cartea and Velasco, 2008). Reducing the seed glucosinolate content (SGC) to the Canola-quality level of <30 μmoles per gram of dry seed weight (DW) has been a major breeding objective in rapeseed and mustard (Potts *et al.*, 1999; Raymer, 2002; So and Duncan, 2021).

All the area under rapeseed, mostly in the sub-temperate regions of the world, is sown with Canola quality varieties and hybrids. Low glucosinolate content in all parts of the Canola quality lines makes them vulnerable to pests and requires the application of pesticides for the protection of the crop (Milovac *et al.*, 2017; Sekulic and Rempel, 2016; Zheng *et al.*, 2020). The increasing cost and restrictions on the use of some of the insecticides, particularly in Europe, and declining insecticide efficacy have threatened the overall production of rapeseed, worldwide (Insecticide Resistance Action Group, 2019).

Mustard is mostly grown in the Indian subcontinent in semi-arid agroecologies and is a potential crop for many other regions with limited water availability. Canola quality low glucosinolate lines have been available in mustard for quite some time (Burton *et al.*, 2004; Love *et al.*, 1990; Ramchiary *et al.*, 2007). However, large-scale cultivation of these lines has not been possible in the Indian subcontinent due to their higher vulnerability to some of the known insect pests of mustard and some generalist pests that have not been reported to feed on the high glucosinolate varieties.

Glucosinolates and their hydrolysis products are the key arsenals of Brassicaceae family members against invading pests and pathogens (Clay *et al.*, 2009; Halkier and Gershenzon, 2006; Hopkins *et al.*, 2009). While lowering the SGC for increasing the nutritional value of the *Brassica* seed meal, a concomitant reduction of glucosinolates in the whole plant needs to be avoided. Pioneering work on the model species *Arabidopsis thaliana* showed that glucosinolates are synthesized in leaves and silique walls (source tissues) and translocated to the seeds (sink

tissues) through the involvement of two related but distinct glucosinolate transporters, GTR1 and GTR2 (Nour-Eldin *et al*., 2012). Analysis of the glucosinolate content of seeds from *gtr1* and *gtr2* single mutants showed a 48 ± 11% reduction in total glucosinolate levels in seeds of the *gtr2* mutant, whereas the seed glucosinolate content in the *gtr1* mutant was not significantly different from that in the wild-type plants. However, the total glucosinolate content of seeds in the *gtr1gtr2* double mutant was reduced to below detection limits. The loss-of-function of *GTR1* and *GTR2* was accompanied by approximately 10-fold increased accumulation of methionine-derived aliphatic glucosinolates in the leaves and silique walls and highly altered distribution of glucosinolates within the leaf (Madsen *et al*., 2014; Nour-Eldin *et al*., 2012; Xu *et al*., 2019). Additionally, the *gtr1gtr2* double mutant plants exhibited morphological penalties, reduced seed size and seed weight compared to the wild-type seeds.

In comparison to *A. thaliana*, the allopolyploid *B. juncea* (AABB) and *B. napus* (AACC) contain multiple homologues of both *GTR1* and *GTR2* genes (Nour-Eldin *et al*., 2017; Tan *et al*., 2022). The genome of *B. juncea* contains six homologues of *GTR1* and a similar number of *GTR2* homologues (Nambiar *et al*., 2021). Nour-Eldin *et al*. (2017) developed *B. juncea* lines containing mutations by TILLING in four out of the six *GTR2* homologues. The four gene mutant contained 44 ± 11 μmoles/g DW of glucosinolate in the seeds, a 60% reduction from the content in the wild-type seeds, which was more than the Canola quality level of 30 μmoles/g DW. However, no information was provided on the leaf and silique wall glucosinolate content, morphology, and agronomic performance of the *GTR2* quadruple mutants.

In two recent reports, *GTR2* homologues of *B. napus* have been mutated by CRISPR/Cas9-based gene editing. Tan *et al*. (2022) identified *BnaC02.GTR2* by association mapping as the key homologue regulating seeds glucosinolate transport. Editing of *BnaC02.GTR2* along with three other *GTR2* homologues resulted in a significant lowering of the SGC. However, null mutations in the four *GTR2* homologues also resulted in a significant reduction in the leaf glucosinolate content (LGC), 1000 seed weight, seed size, changes in amino acid and sugar accumulation, and fatty acid composition in the seeds. In contrast, He *et al*. (2022) showed that loss-of-function mutation in the sole *BnaA06.GTR2* (an orthologue of the *BnaC02.GTR2* mutated by Tan *et al*., 2022) could significantly reduce the SGC; however, no information was provided on the LGC. A significantly lower-than-normal amount of glucosinolate content was observed in the developing silique walls of the three progeny plants of the *GTR2*-mutated *B. napus* line. However, the two studies where a single *GTR2* gene or four different *GTR2* homologues were mutated did not provide the desired low SGC, high LGC plants with normal growth and yield.

Based on the earlier work on glucosinolate transporters in *A. thaliana* and the polyploid nature of *B. juncea*, we initiated our study with the hypothesis that both *GTR1* and *GTR2* family genes will have to be mutated to develop low SGC lines in *B. juncea*. Whether such plants will have normal glucosinolate levels in other parts of the plant for an uncompromised defence and normal yield was an open question. We used the CRISPR/Cas9 system for targeted editing of multiple *BjuGTR1* and *BjuGTR2* homologues in *B. juncea* to generate lines that contained SGC below the Canola quality threshold (<30 μmoles/g DW). The transgene-free *GTR*-edited low SGC lines were identified and evaluated for tissue-specific glucosinolate accumulation and distribution in source tissue, seed yield-influencing traits, and defence against a major necrotrophic pathogen of mustard—*Sclerotinia sclerotiorum* and a generalist pest—*Spodoptera litura*. Our report presents a breakthrough in the breeding of oilseed mustard for improved oil and seed-meal quality without compromising the protection provided by glucosinolates to the plant.

Results

Selection of gRNAs targeting multiple *BjuGTR1* and *BjuGTR2* homologues and development of *GTR*-edited *B. juncea* lines

A high-seed glucosinolate containing *B. juncea* line Varuna, a mega variety belonging to the Indian gene pool of mustard, was selected for gene editing. The genome of *B. juncea* cv. Varuna contains six functional homologues each of the *GTR1* and *GTR2* genes (Nambiar *et al*., 2021; Paritosh *et al*., 2021). To achieve a strong reduction of SGC, we aimed for the simultaneous targeting of most of the *BjuGTR1* and *BjuGTR2* homologues. Scanning of *BjuGTR* homologues in CRISPR-P V2.0 identified a highly conserved 20 nucleotide (nt) region, from where three guide RNAs (gRNA1, gRNA2, and gRNA3) were designed, which altogether could target nine *BjuGTR* homologues with 100% similarity while displaying one-two nt mismatch with *BjuGTR2-B3* homologue (Figure S1). *BjuGTR1-A3* and *BjuGTR1-B3* showing sequence divergence in their corresponding Protospacer Adjacent Motif (PAM) sites (AGG to AGA) were not considered for editing in the study as their expression levels were found to be relatively lower than the other *BjuGTR1* homologues (Nambiar *et al*., 2021).

The target sites for gRNA1, gRNA2, and gRNA3 mapped to the second exon of their respective *BjuGTRs* (Figure 1a), which corresponds to the second transmembrane domain in the encoded BjuGTR proteins (Figure S1). The three 20 nt sgRNAs (PtAtU6-26-gRNA-scaffold) fragments targeting *BjuGTR1* and *BjuGTR2* homologues were amplified separately (Figure S2) and cloned in a PtCsVMV-*SpCas9* containing binary vector to develop GTR1::GTR2(GEd) construct (Figure 1b). *B. juncea* cv. Varuna was transformed following an *Agrobacterium*-mediated genetic transformation protocol described earlier (Augustine *et al*., 2013). A total of 37 primary transformation events (hitherto referred to as the T0 generation) were generated, and the seed glucosinolate content (SGC) in the T1 seeds, collected from each T0 event was estimated using HPLC. The SGC in the T1 seeds of GTR1::GTR2(GEd) lines ranged from 6.21 to 145.88 μmoles/g DW compared to 146.09 ± 4.59 μmoles/g DW present in the wild-type (WT) Varuna (Figure 2a; Table S1). Thirty out of 37 T0 events displayed a significant reduction in the SGC, bringing it at par or lower than the Canola quality standard of <30 μmoles/g DW. All the major aliphatic glucosinolate fractions (sinigrin, gluconapin, and glucobrassicanapin) were found to be significantly reduced in the T1 seeds of the GTR1::GTR2(GEd) events compared to the WT Varuna (Table S1).

Low SGC events exhibited loss-of-function mutations in multiple *BjuGTR1* and *BjuGTR2* homologues

A strong reduction of SGC in GTR1::GTR2(GEd) events prompted us to screen the CRISPR/Cas9-induced mutations in the target *BjuGTR* homologues. A total of 22 representative T0 events were selected for the screening of mutations in the *BjuGTR1* and *BjuGTR2* homologues. Primers specific to each homologue were designed to amplify the DNA segments spanning the gRNA target site and the DNA sequence chromatogram of the amplicon was

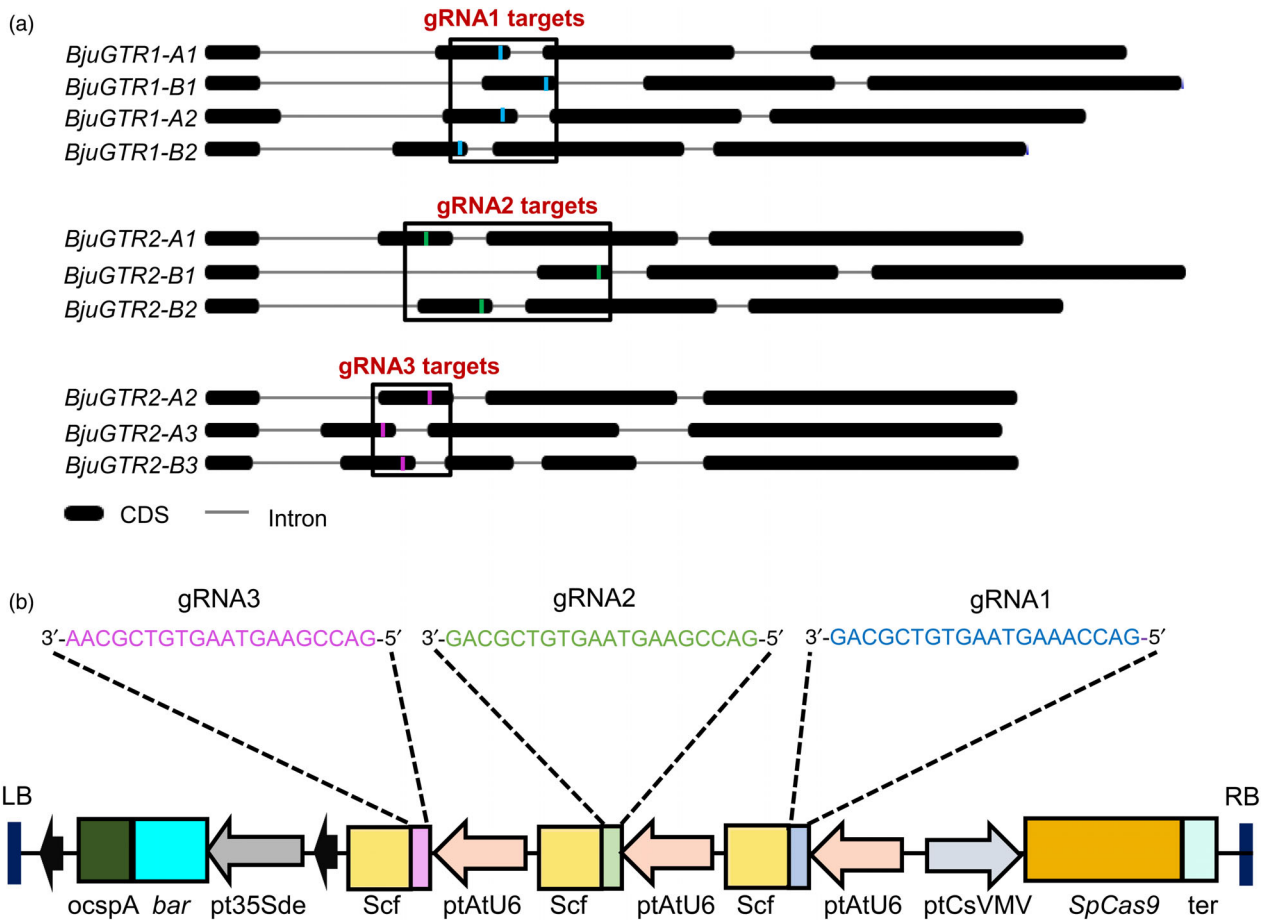


Figure 1 Genomic location of gRNA target sites and generation of GTR1::GTR2(GEd) construct. (a) Genomic localization of target sites of three gRNA viz., gRNA1 (targeting *BjuGTR1-A1*, *BjuGTR1-B1*, *BjuGTR1-A2*, and *BjuGTR1-B2*), gRNA2 (targeting *BjuGTR2-A1*, *BjuGTR2-B1*, and *BjuGTR2-B2*), and gRNA3 (targeting *BjuGTR2-A2*, *BjuGTR2-A3*, and *BjuGTR2-B3*) were designed from the second exon of the target *BjuGTR* genes. (b) T-DNA map showing SpCas9-based BjuGTR1::GTR2(GEd) transformation construct used for targeting the *BjuGTR*s in *B. juncea* cv. Varuna. The three sgRNA fragments containing gRNA1, gNRA2, and gRNA3 were cloned sequentially into the multiple cloning sites of pZP200debar:SpCas9 vector, containing the *bar* gene as the plant selection marker.

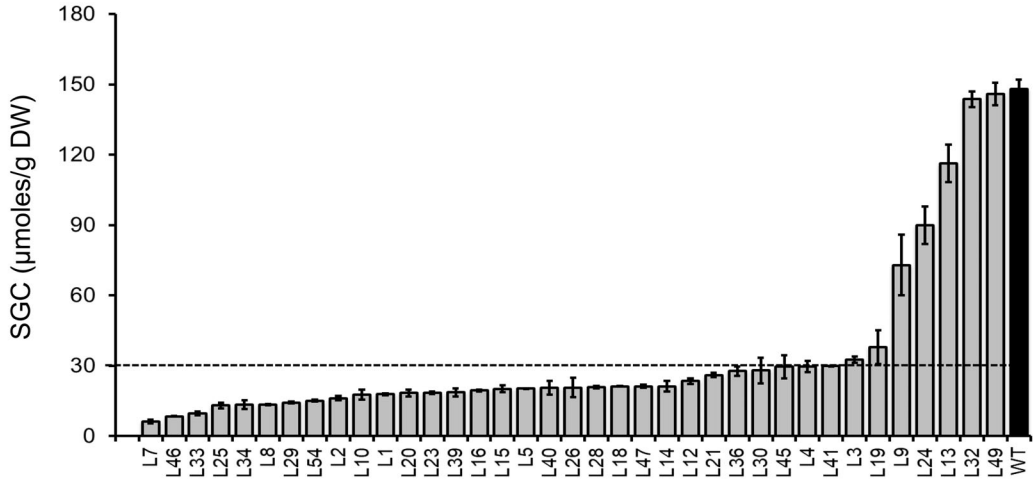
used to determine the mutation efficiency, mutation types, and the zygosity of mutations observed for each *BjuGTR* homologue using the ICE analysis tool. In all the 22 T0 events, the editing frequency ranged from 86% to 100% for the nine *BjuGTR* homologues, showing an overall mutation efficiency of >84% (Figure 2b). The *BjuGTR2-B3* displayed the lowest mutation frequency (31.81%), which could be due to its mismatches with the three gRNAs used. As is the characteristic of CRISPR/Cas9, all the mutations detected were 3–4 nt upstream of the PAM site. Around 90% of the mutations were found to be single nucleotide insertions leading to frame-shift mutations (Figure S3, Table S2). Biallelic homozygous (BHo) and biallelic heterozygous (BHt) were the most frequent mutations observed in the *BjuGTRs* in the T0 events.

The mutation profile of all the *BjuGTR* homologues was compared in T0 events having low SGC (17 events with <30 μmoles/g DW) and high SGC (five events with >70 μmoles/g DW). Sequence analysis suggested that the lines with low SGC exhibited frame-shift mutations in both the alleles of most of the target *BjuGTR1* and *BjuGTR2* homologues, leading to a complete abolishment of the gene function (Figure 2c; Table S2). In the T0 events containing high SGC (L9, L24, L13, L32, and L49), most of the *BjuGTR2* homologues remained either unedited or were in monoallelic (Mo)-edited condition (Figure 2c). Mutation analysis thus suggested that simultaneous editing of multiple *BjuGTR1* and *BjuGTR2* homologues led to low SGC meeting the Canola quality standard.

The low SGC trait and CRISPR/Cas9-induced mutations were inherited stably in the transgene-free *GTR*-edited lines

The inheritance of low SGC trait was analysed in the T1 generation plants of 17 T0 events having low SGC. To identify the transgene-free (*Cas9*-free) plants, the T1 progenies were tested by painting the herbicide 'Basta' on the young leaves followed by PCR analysis for the *bar* gene (Figure S4, Table S3). Based on 'Basta' sensitivity analysis, transgene-free T1 progenies could be identified for most of the low SGC events. A total of 270 T1 progenies were analysed for SGC in the T2 seeds, and almost all progenies showed SGC <30 μmoles/g DW (Table S4). The low SGC trait was found to be stably inherited in the transgene-free lines, with some lines having SGC as low as 3.40 μmoles/g DW in

(a)



(b)

BjuGTR homologues	No. of T0 plants analysed	Mutation frequency (%)	Average mutation efficiency (%)
BjuGTR1-A1	22	100.00	89.91
BjuGTR1-B1	22	100.00	93.45
BjuGTR1-A2	22	95.45	93.43
BjuGTR1-B2	22	100.00	94.45
BjuGTR2-A1	22	100.00	88.00
BjuGTR2-B1	22	100.00	84.33
BjuGTR2-A2	22	90.90	93.81
BjuGTR2-B2	22	95.45	90.57
BjuGTR2-A3	22	86.36	86.89
BjuGTR2-B3	22	31.81	68.00

(c)

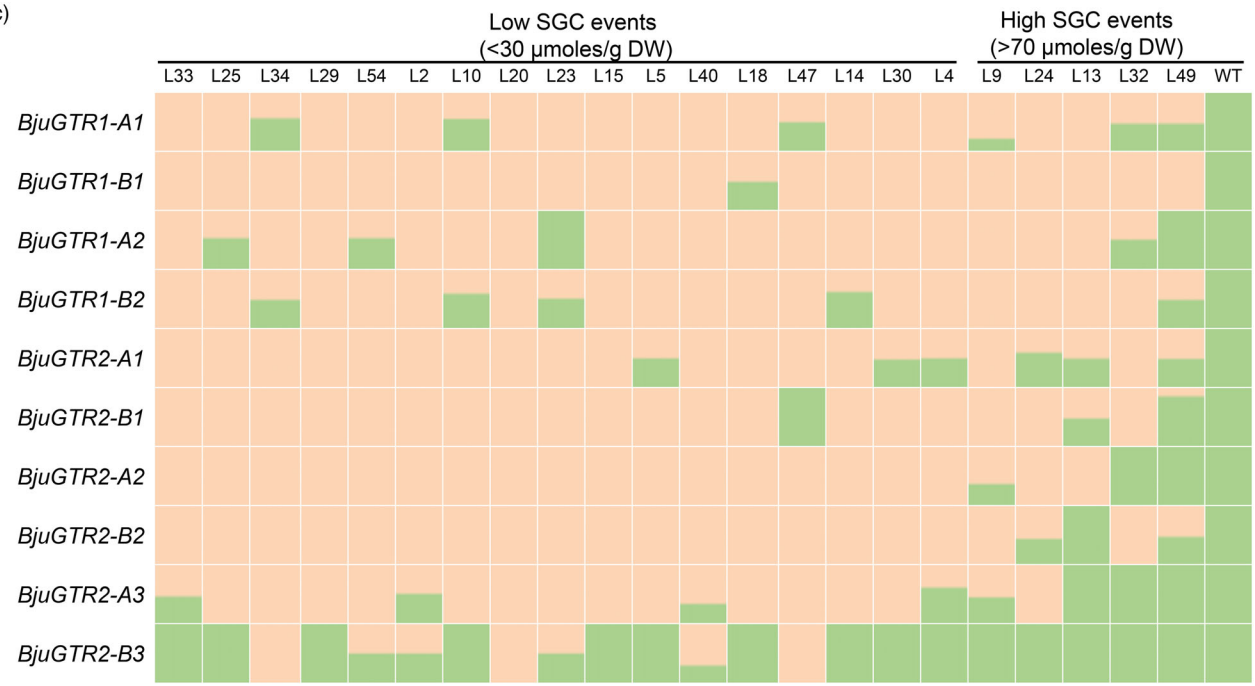


Figure 2 Glucosinolate content and mutation analysis of *GTR*-edited *B. juncea* lines. (a) Comparison of mean seed glucosinolate content (SGC) in T1 seeds (µmoles/g DW) of the 37 primary transgenic (T0) events generated using BjuGTR1::GTR2(GEd) construct and in wild-type (WT) Varuna. The dotted line represents the Canola quality limit of 30 µmoles/g DW. The amounts of total and individual glucosinolate fractions in T1 seeds are provided in Table S1. (b) Mutation frequency and mutation efficiency recorded in different *BjuGTR1* and *BjuGTR2* homologues of 22 T0 events. The analysis was performed using the ICE analysis tool v3.0 and data are provided in Table S2. (c) Analysis of *BjuGTR* editing pattern and genetic zygosity of the CRISPR/Cas9-induced mutations in 22 *GTR*-edited T0 events. Different colour coding shows the functionality (based on mutation) in both the alleles of each *BjuGTR* homologue. The true BHo, and BHt are marked in orange; unedited (wt/wt) alleles are in solid green; and a gradient of orange and green represents the case where one of the alleles remained unedited (Mo) or has an in-frame mutation while reflecting the mutation efficiency.

their T2 seeds. Overall, the mean SGC in the transgene-free *GTR*-edited lines ranged from 17.72 to 30.46 μmoles/g DW in the T3 seeds, all below the Canola quality threshold (Figure 3a; Table S5).

The lower SGC observed in the *GTR*-edited lines led us to perform a detailed mutation analysis in the transgene-free T2 generation plants. A comparison of mutations between T0 and T2 plants suggested that mutations in the ten *BjuGTR* homologues had acquired BHo status in the advanced generation plants, suggesting that the CRISPR/Cas9-induced mutations followed a Mendelian inheritance pattern (Tables S2 and S6). A total of 14 transgene-free low SGC lines with distinct mutations in the *BjuGTR1* and *BjuGTR2* homologues were selected in the T2 generation and maintained by strict self-pollination (Figure 3b). While most of the selected low SGC lines had loss-of-function mutation alleles in *BjuGTR1* and *BjuGTR2* homologues, a few low SGC lines contained in-frame mutations (shorter deletion in the multiple of 3 nt) in a few *BjuGTR1* (L18-6, L18-16, L34-3, L34-4, and L47-1) homologues, suggesting that these lines could have retained the GTR1 functionality in the edited lines. A few low SGC lines (L47-1, L47-3, and L47-6) also contained in-frame mutations in the *BjuGTR2-B1* homologue. The CRISPR/Cas9-induced mutations in the target *BjuGTR1* and *BjuGTR2* homologues of two representative *GTR*-edited low SGC lines are presented in Figure 3c,d.

GTR-edited lines displayed distinct glucosinolate profiles in source and sink tissues during the early vegetative stage

GTR1 and GTR2 have been shown in *A. thaliana* to be involved in the bidirectional transport of glucosinolates during early vegetative growth from leaves to roots and vice versa (Andersen *et al.*, 2013; Chen *et al.*, 2001). To investigate the effect of *BjuGTR* editing on long-distance transport of glucosinolates, the leaf and root glucosinolate content, and profile were analysed in 30-day-old plants of the 14 *GTR*-edited low SGC T2 lines and compared with the content and profile in WT Varuna. The total leaf glucosinolate content (LGC) of *GTR*-edited lines ranged from 69.12 to 92.34 μmoles/g DW which was similar to or higher than the LGC of WT Varuna (68.70 ± 2.67 μmoles/g DW) (Figure 4a). The concentration of the predominant aliphatic glucosinolates as well as indolic and benzenic glucosinolates was found to be increased in the young leaf of the *GTR*-edited low SGC lines (Table S7).

A somewhat higher accumulation of leaf glucosinolates in young leaves prompted us to compare the root glucosinolate profile of the *GTR*-edited lines with that of the WT Varuna, in 30-day-old plants. The total root glucosinolate content (RGC) in *GTR*-edited lines ranged from 24.94 to 40.67 μmoles/g DW, somewhat similar to that present in the WT roots (40.45 ± 1.71 μmoles/g DW) (Figure 4b). However, when individual glucosinolate fractions were analysed, it was found that both aliphatic and indolic glucosinolate pools were reduced by 0.22–0.75 folds in the roots of the *GTR*-edited lines compared to the WT plant (Table S8). Interestingly, the root-predominant benzenic glucosinolate—gluconastrutiin (NAS) was found to have a concomitant increase by 1.03–2.05 folds in the *GTR*-edited low SGC lines over the level recorded in the WT roots (14.97 ± 1.32 μmoles/g DW), thereby compensating for the total RGC in the *GTR*-edited lines to some extent. It can be concluded that mutations in *BjuGTR1* and *BjuGTR2* homologues have a significant effect on the bidirectional transport of glucosinolates between roots and shoots in the *GTR*-edited *B. juncea* lines.

GTR-edited lines had higher glucosinolate accumulation in source tissues during the reproductive stage

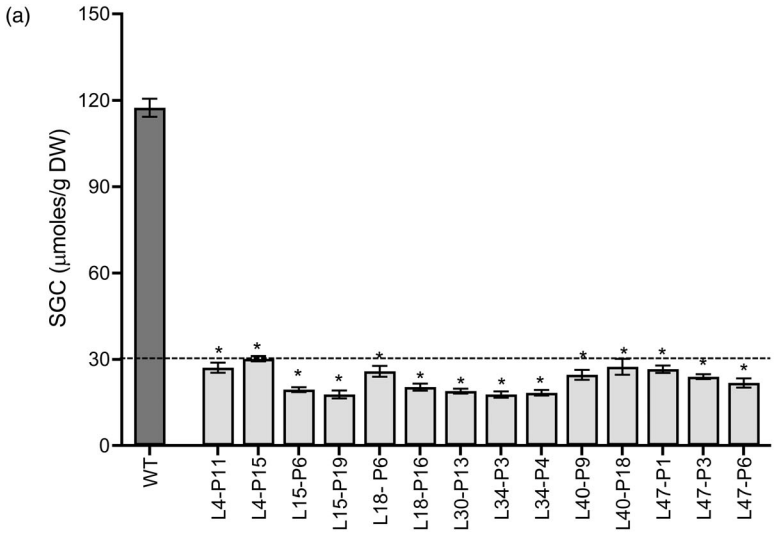
The glucosinolate accumulation in source tissues was estimated again when the plants of the selected events reached the reproductive stage. Glucosinolate content was analysed in the flag leaf of the 14 *GTR*-edited T2 lines, at the 2-month-old stage, and compared with the WT Varuna plants at the same stage. The LGC of the flag leaf in the low SGC lines ranged from 75.84 to 107.71 μmoles/g DW, which was significantly higher than that of the WT Varuna (68.48 ± 0.73 μmoles/g DW), with an over-accumulation of the predominant aliphatic glucosinolates (Figure 4c; Table S9).

The silique walls enclose the developing seeds and protect them from pests and pathogens. Siliques are important source tissues for glucosinolates that are transferred to the developing seeds. We, therefore, estimated glucosinolate content in the silique walls as well as in the developing green seeds of the 14 transgene-free *GTR*-edited T2 lines. The silique wall glucosinolate content (SWGC) of the *GTR*-edited lines ranged from 33.57 to 63.54 μmoles/g DW, which was either similar to or higher than the WT Varuna (37.87 ± 1.36 μmoles/g DW) (Figure 4d; Table S10). The developing green seeds accumulated significantly lower amounts of glucosinolates (gSGC) in all the *GTR*-edited lines compared to the WT plants (Table S11), suggesting an impaired glucosinolate transport from the silique walls to the developing seeds.

GTR-edited lines displayed uneven glucosinolate distribution in mature leaves

GTRs in Arabidopsis have been found to affect the spatial distribution of glucosinolates in the leaves (Madsen *et al.*, 2014). To investigate the effect of *GTR*-editing on glucosinolate distribution in the mature leaves, we picked eight out of 14 *GTR*-edited low SGC T2 lines for detailed analysis. Leaves were dissected across the two horizontal planes viz., the x-axis (margin, lamina, and midrib) and the y-axis (tip, base, and petiole), and glucosinolate concentrations were estimated in these regions using HPLC.

In the WT Varuna leaves, glucosinolates accumulated at almost similar concentrations across the margin, lamina, and midrib suggesting an active glucosinolate transport across the leaf (Figure 5a). In comparison, in the *GTR*-edited low SGC lines, the glucosinolate concentration across the *x*-axis leaf sections was strikingly different from that in the wild-type leaves (Figure 5a; Table S12). Glucosinolate concentration in the margin of *GTR*-edited lines ranged from 164.10 to 226.54 μmoles/g DW, significantly higher than in the WT Varuna (53.21 ± 6.08 μmoles/g DW). We observed an interesting trend wherein glucosinolate concentration in the margin of *GTR*-edited lines having in-frame mutations in at least one *BjuGTR1* homologue (L18-16, L34-3, and L34-4; hereafter referred to as Category-I (Ct-I) lines) ranged from 190.22 to 226.54 μmoles/g DW, which was somewhat higher than the lines having mutations in all the four *BjuGTR1* homologues (L4-11, L4-15, L15-19, L30-13, and L40-9; referred to as Ct-II lines) with glucosinolates ranging from 160.10 to 190.95 μmoles/g DW (Figure 5a). Similarly, the glucosinolate concentration in leaf lamina in Ct-I (147.70–189.20 μmoles/g DW) lines was significantly higher than the Ct-



(b)

	BjuGTR1						*BjuGTR2*					
	-A1	*-B1*	*-A2*	*-B2*	*-A3*	*-B3*	*-A1*	*-B1*	*-A2*	*-B2*	*-A3*	*-B3*
L4-11	+1 / +1	+1 / +1	−10 / −10	+1 / +1	0 / 0	0 / 0	+1 / +1	+1 / +1	−5 / −5	+1 / +1	+1 / 0	0 / 0
L4-15	+1 / +1	+1 / +1	+1 / +1	+1 / +1	0 / 0	0 / 0	−13 / −13	+1 / +1	+1 / +1	+1 / +1	0 / 0	0 / 0
L15-6	+1 / +1	+1 / +1	+1 / +1	−1 / −1	0 / 0	0 / 0	−2 / −2	+1 / +1	+1 / +1	+1 / +1	+1 / +1	0 / 0
L15-19	+1 / +1	+1 / +1	+1 / +1	+1 / +1	0 / 0	0 / 0	−2 / −2	+1 / +1	+1 / +1	+1 / +1	+1 / +1	0 / 0
L18-6	+1 / +1	−3 / −3	+1 / +1	+1 / +1	0 / 0	0 / 0	−1 / −1	−1 / −1	−2 / −2	+1 / +1	+1 / +1	0 / 0
L18-16	+1 / +1	−3 / −3	+1 / +1	+1 / +1	0 / 0	0 / 0	+1 / −1	−19/−1	+1 / −2	+1 / +1	+1 / +1	0 / 0
L30-13	+1 / +1	+1 / +1	+1 / +1	+1 / +1	0 / 0	0 / 0	+1 / +1	+1 / +1	−4 / −4	+1 / +1	+1 / +1	0 / 0
L34-3	−24 / −24	+1 / +1	+1 / +1	−6 / −6	0 / 0	0 / 0	+1 / +1	+1 / +1	+1 / +1	+1 / +1	+1 / +1	−4 / −4
L34-4	−24 / −24	+1 / +1	+1 / +1	+1 / +1	0 / 0	0 / 0	+1 / +1	+1 / +1	+1 / +1	+1 / +1	+1 / +1	−4 / −4
L40-9	+1 / +1	+1 / +1	+1 / +1	+1 / +1	0 / 0	0 / 0	+1 / +1	+1 / +1	+1 / +1	+1 / +1	+1 / 0	−2 / −2
L40-18	+1 / +1	+1 / +1	+1 / +1	+1 / +1	0 / 0	0 / 0	+1 / +1	+1 / +1	+1 / −17	+1 / +1	+1 / +1	−2 / −2
L47-1	−3 / −3	−13 / +1	+1 / +1	+1 / +1	0 / 0	0 / 0	+1 / +1	−39 / −39	+1 / −2	+1 / −2	+1 / −13	+1 / +1
L47-3	+1 / +1	−13 / +1	+1 / +1	+1 / +1	0 / 0	0 / 0	+1 / +1	−39 / −3	+1 / −2	+1 / −2	−13 / −13	+1 / +1
L47-6	+1 / +1	−13 / +1	+1 / +1	+1 / +1	0 / 0	0 / 0	+1 / +1	−39 / −3	+1 / −2	+1 / −2	−13 / −13	+1 / +1
WT	0/ 0	0/ 0	0/ 0	0/ 0	0 / 0	0 / 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0	0/ 0

(c)

BjuGTR1

-A1

```
WT      CCTCTGCGA-CACTTACTTTGGTCGCTA
L15-19  CCTCTGACGCCACTTACTTTGGTCGCTA   +1
L18-6   CCTCTGACGCCACTTACTTTGGTCGCTA   +1
```

-B1

```
WT      CCTCTG-CGACACTTACTTTGGTCGCTA
L15-19  CCTCTGACGACACTTACTTTGGTCGCTA   +1
L18-6   CCTCTG----CACTTACTTTGGTCGCTA   -3
```

-A2

```
WT      CCTCTG-CGACACTTACTTTGGTCGCTA
L15-19  CCTCTGACGACACTTACTTTGGTCGCTA   +1
L18-6   CCTCTGACGACACTTACTTTGGTCGCTA   +1
```

-B2

```
WT      CCTCTG-CGACACTTACTTTGGTCGCTA
L15-19  CCTCTGACGACACTTACTTTGGTCGCTA   +1
L18-6   CCTCTGACGACACTTACTTTGGTCGCTA   +1
```

(d)

BjuGTR2

-A1

```
WT      CCTCTGCGACACTTACTTCGGTCGATAC
L15-19  CCTCTG--ACACTTACTTCGGTCGATAC   -2
L18-6   CCTCTG-CACACTTACTTCGGTCGATAC   -1
```

-B1

```
WT      CCTCTG-CGACACTTACTTCGGTCGATAC
L15-19  CCTCTGACGACACTTACTTCGGTCGATAC   +1
L18-6   CCTCTG--GACACTTACTTCGGTCGATAC   -1
```

-A2

```
WT      CCTTTG-CGACACTTACTTCGGTCGTTAC
L15-19  CCTTTGACGACACTTACTTCGGTCGTTAC   +1
L18-6   CCTTTG---ACACTTACTTCGGTCGTTAC   -2
```

-B2

```
WT      CCTCTG-CGACACTTACTTCGGTCGTTAC
L15-19  CCTCTGACGACACTTACTTCGGTCGTTAC   +1
L18-6   CCTCTGTGACACTTACTTCGGTCGTTTAC   +1
```

Figure 3 Analysis of the 14 transgene-free *GTR*-edited low SGC *B. juncea* lines in T2 generation. (a) Mean glucosinolate content in T3 seeds of 14 *GTR*-edited lines along with wild-type (WT) Varuna. Mature seeds obtained from 8 to 12 T2 plants of each transgene-free event were analysed for glucosinolate content (µmoles/g DW) using HPLC. The dotted line represents the Canola quality limit of 30 µmoles/g DW. Asterisk indicates statistically significant differences between *GTR*-edited lines and WT seeds (two-tailed Student's t-test, $P \leq 0.05$). The individual glucosinolate data are provided in Table S5. (b) Mutation pattern and genetic zygosity of the CRISPR/Cas9-induced mutations in 14 *GTR*-edited T2 plants. Different colour coding shows the functionality of alleles for each *BjuGTR* homologue. The BHo and BHt mutations are marked in orange, unedited (wt/wt) alleles in solid green, and gradient of orange/green are the cases where one of the alleles is either unedited (Mo) or has an in-frame mutation. '+' and '−' denote the number of nucleotides inserted or deleted in both alleles. The mutation data in T2 lines are provided in Table S6. Sequence alignment showing the mutations at the target sites of well-expressed (c) *BjuGTR1* and (d) *BjuGTR2* homologues of two representative *GTR*-edited low SGC lines.

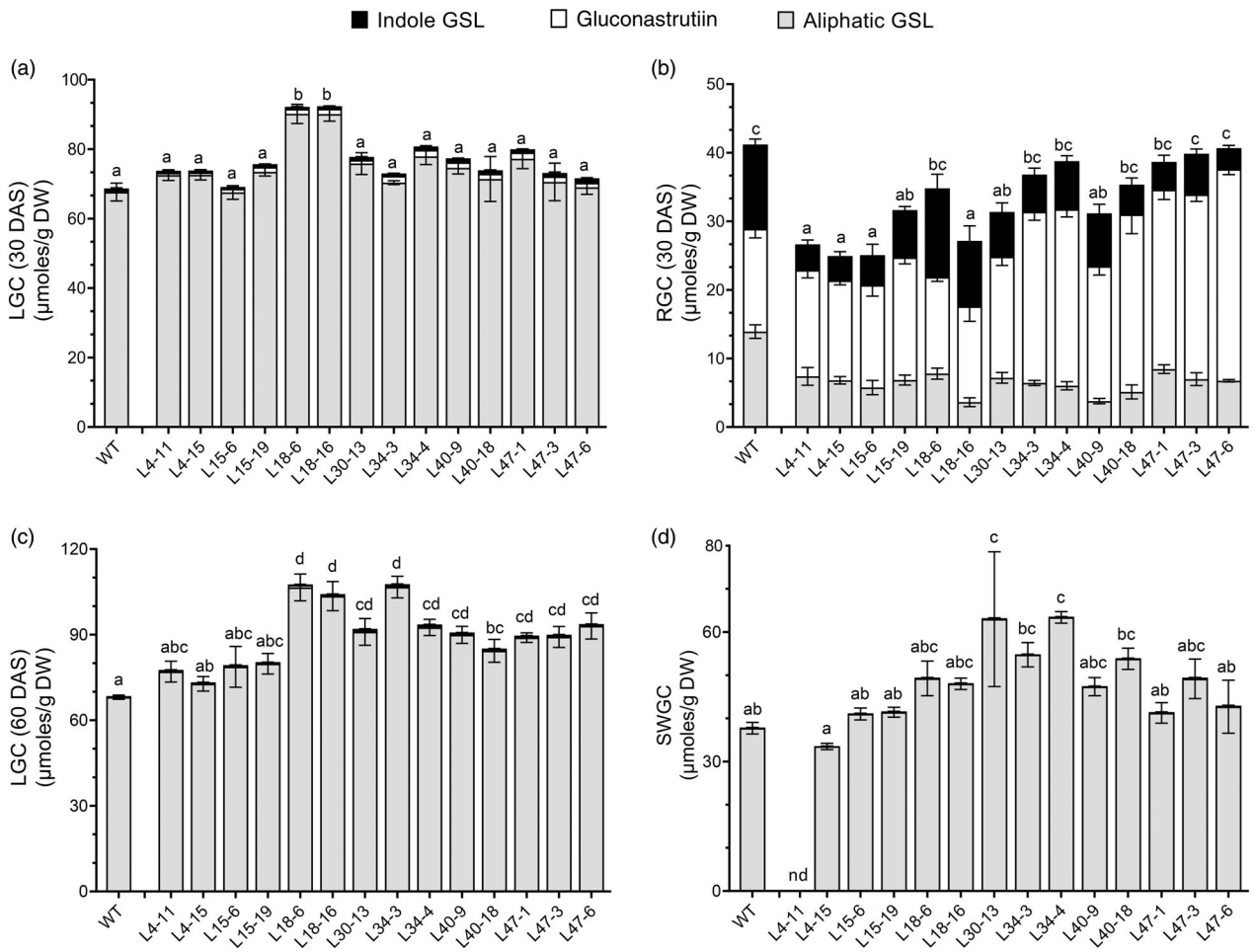


Figure 4 Comparison of glucosinolate content and profile in different developmental tissues of the 14 transgene-free *GTR*-edited *B. juncea* lines. Bar plot showing the mean glucosinolate content and profile (µmoles/g DW) in (a) young leaves (1-month-old plant); (b) root (1-month-old plant); (c) flag leaf (2-month-old plant); and (d) Silique wall of the *GTR*-edited lines and wild-type (WT) Varuna plants. A total of 4–10 T2 plants were analysed and each bar represents the mean glucosinolate content ±SE estimated using HPLC. Different letters on top of the bars indicate significant differences in glucosinolate content using *Tukey's post hoc* test at $P \leq 0.05$. Glucosinolate data in the young leaf, root, flag leaf, and silique wall are provided in Tables S7–S10 respectively. GSL, glucosinolate; DAS, days after sowing.

II (133.65–159.42 µmoles/g DW) lines and compared to that in the WT plants (64.61 ± 7.07 µmoles/g DW) (Figure 5a; Table S12). In the leaf midrib, a marginal over-accumulation of glucosinolate concentration was observed in the Ct-I lines compared to the Ct-II lines and WT plants (Figure 5a; Table S12).

In the WT Varuna leaf, glucosinolates were distributed evenly across the tip and base, whereas the petiole had significantly lower glucosinolate concentration (Figure 5b). In comparison, the glucosinolate concentration in the *GTR*-edited lines was found to be highest at the leaf tip followed by the base and the petiole (Table S13). The glucosinolate concentration in the tip of the Ct-I lines (ranged from 184.95 to 209.43 µmoles/g DW) and Ct-II lines (147.90–187.39 µmoles/g DW) were significantly higher than that of the WT Varuna (72.20 ± 2.55 µmoles/g DW) (Figure 5b). Similarly, glucosinolate concentrations in the leaf base of Ct-I (164.80–183.38 µmoles/g DW) and Ct-II (137.50–185.45 µmoles/g DW) lines were significantly higher compared to that in the WT Varuna plants (79.77 ± 4.29 µmoles/g DW) (Figure 5b; Table S13). The glucosinolate concentration in the petiole of *GTR*-edited Ct-I and Ct-II lines was almost similar to the WT plants (Figure 5b; Table S13). Thus, mutations in *BjuGTR1* and *BjuGTR2* homologues seem to have a significant effect on leaf glucosinolate distribution in the *GTR*-edited *B. juncea* lines.

GTR-edited low-seed, high-leaf glucosinolate lines had uncompromised defence response

The performance of transgene-free *GTR*-edited low-seed, high-leaf glucosinolate lines, and WT Varuna was tested against a broad-host range necrotrophic pathogen *S. sclerotiorum* and a generalist pest *Spodoptera litura*. Low-leaf, low-seed glucosinolate Varuna lines (DH-3325 and DH-2901) developed by marker-assisted breeding (Bisht *et al.*, 2009; Ramchiary *et al.*, 2007) and a BjuMYB28-RNAi line C3/15 developed earlier in our laboratory (Augustine *et al.*, 2013) served as controls. Detached young leaves from 1-month-old plants were used for the pathogen and pest assays under controlled conditions.

Forty-eight hours post S. *sclerotiorum* inoculation, the Canola quality DH lines (LGC ranging from 0.25 to 0.34 µmoles/g DW) and BjuMYB28-RNAi line (LGC 8.73 ± 0.94 µmoles/g DW) had significantly larger lesions as compared to the *GTR*-edited lines (LGC ranging from 65.30 to 92.32 µmoles/g DW) and WT Varuna (LGC 67.42 ± 1.45 µmoles/g DW) (Figure 6a). The lesion areas in

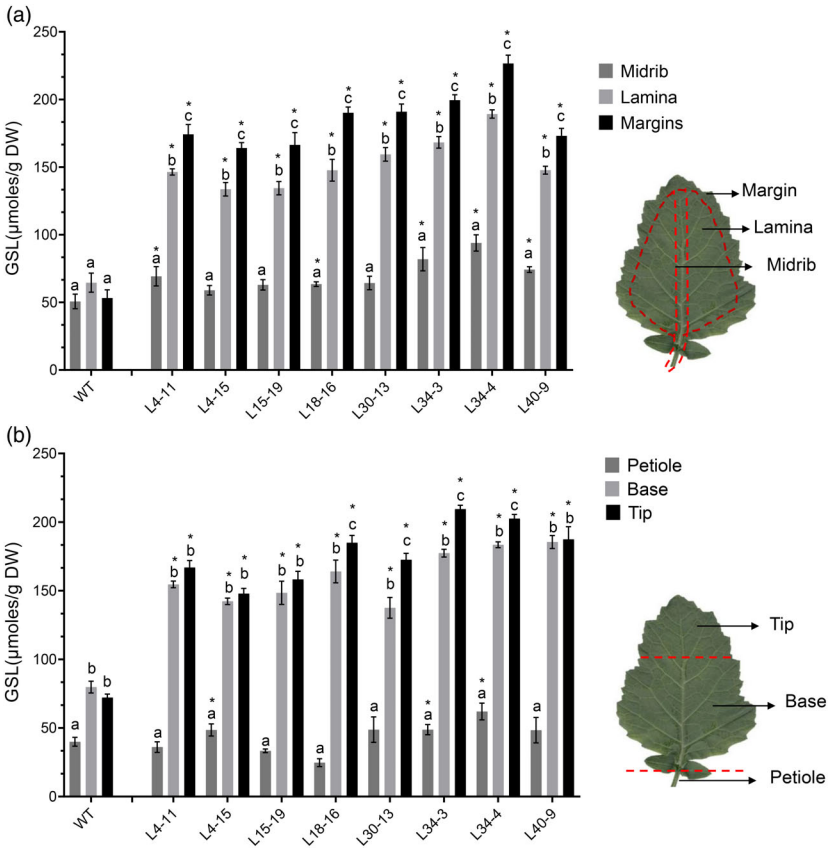


Figure 5 Glucosinolate distribution in mature leaves of *GTR*-edited low SGC *B. juncea* lines. The mature leaf of eight representative *GTR*-edited low SGC lines and wild-type (WT) Varuna was dissected across the two horizontal planes viz., the (a) *x*-axis (margin, lamina, and midrib) and (b) *y*-axis (tip, base, and petiole) planes and glucosinolate concentrations (μmoles/g DW) were estimated using HPLC. Dashed lines on the leaf photograph depict the dissected leaf parts. Each bar represents mean glucosinolate concentration and error bars (n = 6). Letters above-grouped columns indicate statistically significant differences within dissected leaves (one-way ANOVA, $P \leq 0.05$). Asterisk indicates statistically significant differences between *GTR*-edited leaf parts and the equivalent WT Varuna leaf part (two-tailed Student's *t*-test, $P \leq 0.05$). For individual glucosinolate data, see Tables S12 and S13.

the *GTR*-edited lines were either comparable to or less than the WT Varuna (Table S14). The uncompromised defence response of the *GTR*-edited lines against the tested pathogen infection was quite in accordance with their LGC. The results showed that the *GTR*-edited lines were similar to the WT plants in their defence response while the low-seed, low-leaf Canola quality lines were more susceptible to the pathogen.

We also tested the performance of the *GTR*-edited lines against a generalist pest, *S. litura* using a no-choice insect feeding assay, and the larval weight gain was determined at 6 and 9 days post-infestation (dpi). At 6 dpi, the larvae fed on the *GTR*-edited lines showed either similar or lower weight gain compared to the WT Varuna leaves; however, *GTR*-edited lines performed significantly better than the low-seed, low-leaf Canola quality breeding lines (Figure 6b; Table S15). This trend remained consistent at 9 dpi, with differences in weight gain of larvae inoculated on Canola quality DH lines becoming more pronounced and statistically significant compared to that of the WT Varuna and *GTR*-edited lines. As with the pathogen, the *GTR*-edited lines had an uncompromised defence against the insect pest.

GTR-edited lines had unaltered seed quality and yield-related traits

The 14 transgene-free low-seed, high-leaf glucosinolate lines were grown in a containment net-house along with the WT Varuna. The major seed yield-related trait namely, the 1000 seed weight (Tsw) of the *GTR*-edited lines was found to be similar to Tsw of the WT Varuna (Figure 7a, Table S16). Mature seeds were tested for various seed traits including oil content, protein content, and fatty acid profile. No significant difference was observed in the total oil content in the WT Varuna and the tested *GTR*-edited lines (Figure 7b). The major fatty acid fractions namely oleic acid (C18:1), linolenic acid (C18:3), and erucic acid (C22:1) were also found to be unaltered in the *GTR*-edited lines compared to the control plants (Table S16). The total protein content of the *GTR*-edited low SGC lines ranged from 22% to 24%, marginally higher than the WT Varuna seeds (Figure 7c). These results together suggest that the *GTR*-edited low-seed, high-leaf glucosinolate lines developed in this study performed at par with the WT Varuna for the tested traits.

Discussion

Glucosinolates are the major defence compounds in species belonging to the Brassicaceae family, including the Brassica species belonging to the U's triangle that are cultivated globally as oilseed and vegetable crops. It is now well-established that seed lacks the glucosinolates biosynthesis machinery and most of the seed-bound glucosinolates are synthesized in reproductive organs including silique walls and rosette (Jørgensen *et al.*, 2015; Xu *et al.*, 2023). The most involved work on the glucosinolate transport mechanism has been carried out on the model plant *A. thaliana* where two genes, *GTR1* and *GTR2*, were shown to be involved with the transport of glucosinolates from source tissues to the seeds (Nour-Eldin *et al.*, 2012). Analysis of glucosinolate content of seeds of Arabidopsis mutants showed that while the *gtr2* mutant could provide a 48 ± 11% reduction in SGC, the *gtr1* mutant did not differ significantly from the WT Col-0 for SGC (Nour-Eldin *et al.*, 2012). However, in the *gtr1gtr2* double mutant, the SGC was reduced dramatically and was below the detection limits.

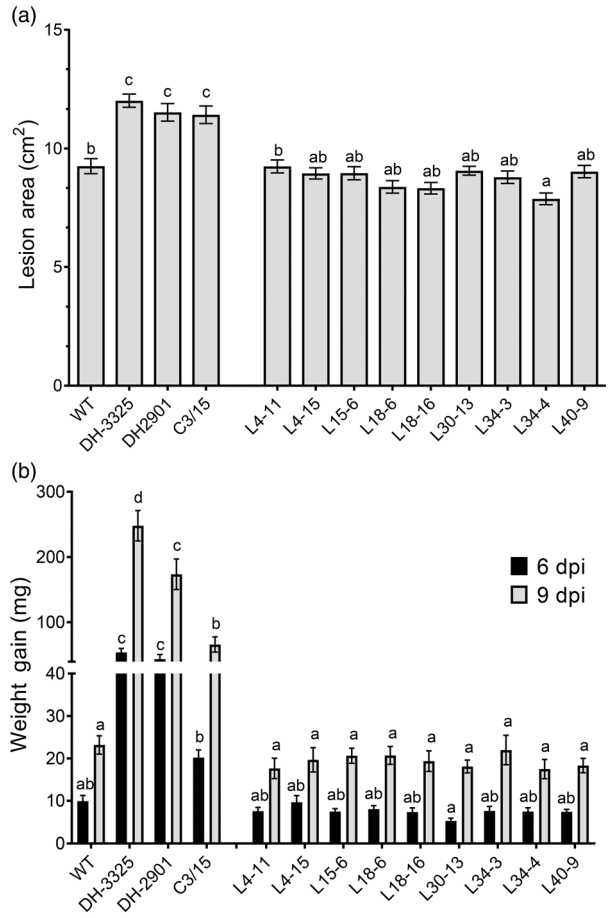


Figure 6 Defence performance of *GTR*-edited low-seed, high-leaf glucosinolate *B. juncea* lines. (a) The detached leaf assays of *GTR*-edited lines against *S. sclerotiorum* under controlled conditions. One-month-old leaves of wild-type (WT) Varuna, *GTR*-edited lines, doubled-haploid lines (DH-3325, DH-2901), and a BjuMYB28-RNAi (C3/15) line were infected with *S. sclerotiorum* and lesion areas (cm^2) were recorded at 48 hpi. Data represent the mean ± SE ($n \geq 14$) of the two independent experiments conducted. Different letters on top of bars indicate significant differences ($P \leq 0.05$) using Tukey's *post hoc* range tests. (b) Performance of generalist pest *S. litura* on *GTR*-edited lines. Young leaves of the tested lines were fed to the second instar larvae and the average weight gain of the larvae was recorded on the sixth and ninth day post-infestation (dpi) in a no-choice experimental design. Data represent the mean ± SE ($n \geq 10$) of two independent experiments. Different letters on top of bars indicate significant differences ($P \leq 0.05$) across the lines for each time point, using Tukey's *post hoc* range tests. The data on pathogen and pest assays is provided in Tables S14 and S15 respectively.

Mutations in the Arabidopsis *GTR1* and *GTR2* genes also resulted in substantial alterations in the glucosinolate distribution pattern in the leaves and other vegetative parts. For instance, the *gtr1gtr2* mutant had low glucosinolate content in stem, roots, and root exudates (Madsen *et al.*, 2014; Xu *et al.*, 2017, 2019), and over-accumulation in the leaf tip (Madsen *et al.*, 2014), cauline leaves (Xu *et al.*, 2019), and silique walls (Nour-Eldin *et al.*, 2012). Furthermore, *gtr1gtr2* mutant plants also exhibited morphological penalties including reduced seed size and seed weight compared to the WT Col-0 plants (Nour-Eldin *et al.*, 2012). In Arabidopsis, it has been shown that the *GTR1* has other accessory roles including the phytohormones-mediated stamen and root development (Kuo *et al.*, 2020; Saito *et al.*, 2015). Thus, fine manipulation of glucosinolate transporters is critical for eliminating the accumulation of anti-nutritional glucosinolates in sink tissues, without affecting the overall distribution of defence metabolite in other plant parts.

In comparison to Arabidopsis, the Brassica species belonging to U's triangle contain multiple homologues of the *GTR1* and *GTR2* genes (Nambiar *et al.*, 2021; Nour-Eldin *et al.*, 2017). It is now well established that the three diploid genomes of Brassica U's triangle—*B. rapa* (AA), *B. nigra* (BB), and *B. oleracea* (CC) are allohexaploids where the three sub-genomes, represented as LF, MF1, and MF2, have undergone differential gene fractionation with LF sub-genome retaining much larger repertoire of genes as compared to the MF1 and MF2 sub-genomes (Cheng *et al.*, 2013; Lysak *et al.*, 2007). However, all three genomes—AA, BB, and CC have retained three homologues of both *GTR1* and *GTR2* genes, whereas the three allopolyploids in the U's triangle—*B. juncea* (AABB), *B. napus* (AACC), and *B. carinata* (BBCC) contain six homologues each of *GTR1* and *GTR2* (Nambiar *et al.*, 2021). Whether these genes have undergone sub-functionalization or neo-functionalization in the extant *Brassica* species is not known.

Since the discovery of glucosinolate transporters in Arabidopsis, limited studies in Brassica crops have used *GTR2* homologue(s) as the key target for engineering the SGC. The loss-of-function mutations (generated through TILLING) in four *GTR2* homologues, singly and in combination, were first tested in *B. juncea* for reducing the SGC; however, lines having SGC within the desired Canola quality level could not be obtained even in the *BjuGTR2* quadruple mutants (Nour-Eldin *et al.*, 2017). Two recent reports in *B. napus* demonstrated that CRISPR/Cas9-generated loss-of-function mutations in one of four *BnaGTR2* homologues were not sufficient to achieve the ideal low-seed, high-leaf glucosinolate phenotype (He *et al.*, 2022; Tan *et al.*, 2022). Further, loss-of-function in *GTR2* homologues was associated with lower glucosinolates levels in leaf and silique walls and impacted various seed metabolite traits negatively in the *BnaGTR2*-edited *B. napus* lines. These studies have unequivocally shown that achieving SGC below the Canola threshold level (<30 μmoles/g DW) is not possible by mutating only a subset of *GTR2* homologues in rapeseed and mustard.

Our study demonstrates that simultaneous editing of multiple homologues of the *B. juncea GTR1* and *GTR2* gene family provides a desirable level of low SGC at par with the Canola quality standard in oilseed mustard. CRISPR/Cas9-based multiplex gene-editing construct used in our study could edit up to ten *BjuGTR* homologues with very high mutation efficiency in the polyploid *B. juncea*. The strategy led to complete loss-of-function mutations in the target *BjuGTR* homologues and the development of low SGC events in the first generation itself. Subsequently, a large number of transgene-free *B. juncea* lines inheriting the CRISPR/Cas9-induced mutations in multiple *BjuGTR1* and *BjuGTR2* homologues could be achieved easily, which is another example of the usefulness of the genome-editing technology (Wolt and Wolf, 2018).

Despite the differences in the gene number, our study shows that there are parallels between Arabidopsis and *B. juncea* while having some differences. The mutations in *BjuGTR1* and *BjuGTR2* homologues provided a marginally increased accumulation of leaf glucosinolates in the early vegetative stage of the *GTR*-edited *B. juncea* lines. However, at the reproductive stage, 1.2–1.5-fold over-accumulation of glucosinolates in the source tissues such as flag leaves and silique walls was quite evident in the *GTR*-edited

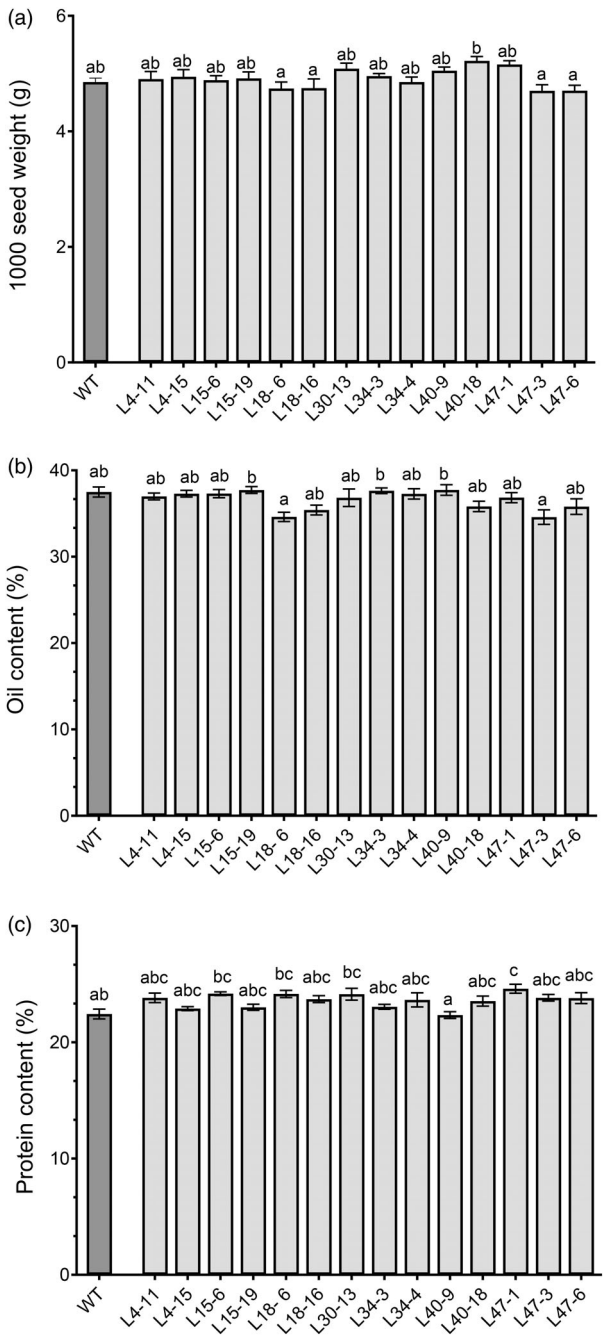


Figure 7 Performance of 14 transgene-free *GTR*-edited low-seed, high-leaf glucosinolate lines for seed yield and quality parameters. Bar plot showing the (a) 1000 seed weight (TSW, in grams), (b) Oil content (in %), and (c) Protein content (in %) in T3 mature seeds of the *GTR*-edited low SGC *B. juncea* lines in comparison with the wild-type (WT) Varuna. The data represent the mean ± SE of measurements from 8 to 12 T2 plants of each transgene-free *GTR*-edited line. Different letters on top of the bars indicate significant differences using *Tukey's post hoc* test at $P \leq 0.05$. The seed yield and quality parameters of *GTR*-edited lines are provided in Table S16.

lines. This was quite in contrast with the Arabidopsis, where *gtr1gtr2* mutants have a 10-fold increased accumulation of methionine-derived aliphatic glucosinolates in the leaves and silique walls (Nour-Eldin *et al.*, 2012). Nonetheless, studies in both Arabidopsis and polyploid *B. juncea* strengthen the fact that the *GTR1* and *GTR2* genes play a critical role in transporting glucosinolates from source tissues (silique walls and flag leaves) to the seeds (sink). Further, our findings are in agreement with a recent study in Arabidopsis which suggests that de novo synthesis and transport within the reproductive tissues are sufficient to supply the seeds with glucosinolates (Xu *et al.*, 2023).

The impaired glucosinolate transport from the source to sink in the *GTR*-edited *B. juncea* lines could turn the source (leaf) into a permanent sink. Consequently, the leaf-synthesized aliphatic and indolic glucosinolates may not be loaded in the phloem for transport to the root, thus explaining their higher accumulation in the leaf with a concomitant reduced accumulation in the roots of the *GTR*-edited lines. A predominantly high concentration of gluconasturtiin (NAS) in the *GTR*-edited lines is an interesting observation. Since the targeted *BjuGTR* homologues are well expressed in the 1-month-old root (Nambiar *et al.*, 2021; Nour-Eldin *et al.*, 2017), the reduced transport of the root-synthesized glucosinolate NAS towards the above-ground tissues in all probability has led to a higher accumulation of NAS in the roots of the *GTR*-edited lines compared to the WT Varuna. The low volatility and hydrophobicity of the breakdown product of NAS (2-phenyl-ethyl-isothiocynate) may make its over-accumulation advantageous for the plant as it could reduce the below-ground herbivore performance (van Dam *et al.*, 2009; van Leur *etal.*, 2008).

Further, in the mature leaf of *GTR*-edited lines, a significantly higher glucosinolate concentration in the leaf tip compared to the petiole as well as in the leaf margin compared to the midrib was also quite evident in our study. Our observations are quite in agreement with the altered leaf glucosinolate concentrations reported for the Arabidopsis *gtr1gtr2* mutant (Madsen *et al.*, 2014), thereby confirming that *GTR1* and *GTR2* are involved in the retention of glucosinolates at the base of the mature leaf and are also required for proper intra-leaf distribution of glucosinolates along the vasculature and outer leaf perimeters (Jørgensen *et al.*, 2015). It seems plausible that due to the impaired BjuGTR1 and BjuGTR2 functions in the *GTR*-edited lines, the leaf-synthesized glucosinolates are not loaded into the phloem and remained in the apoplast of the leaf thereby leading to their increased accumulation in the leaf margin as well as in the leaf tip.

The differential distribution of glucosinolates in the mature leaf of the *GTR*-edited lines, belonging to Ct-I and Ct-II types, is another interesting observation in this study. The distinct mutation profile in *BjuGTR1* homologues between Ct-I and Ct-II lines somewhat explains this variation. It is possible that due to short in-frame mutations in Ct-I lines, a few functional *BjuGTR1* homologues might provide the import of the apoplastic glucosinolates back into the symplasm to some extent while maintaining the intra-leaf glucosinolate distributions in these lines. Nonetheless, the *GTR*-edited lines, having mutations in up to ten *BjuGTR* homologues, can be segregated in the near future work to identify the combinations of *BjuGTR* mutations that are necessary to generate the agronomically viable low-SGC, high-LGC lines with least impact on glucosinolate over-accumulation and distribution in the source tissues.

Our work clearly demonstrates that simultaneous editing of multiple *BjuGTR1* and *BjuGTR2* homologues, but not all, can generate low-SGC, high-LGC mustard lines with uncompromised defence and yield performance. We demonstrated the benefits of the high-leaf glucosinolate content by testing the performance of

GTR-edited lines against a necrotrophic pathogen (*S. sclerotiorum*) and a generalist pest (*S. litura*). The high-leaf glucosinolates in the *GTR*-edited lines helped them to perform better or at par with the WT Varuna against the tested pest and pathogen, while displaying a significant advantage over the Canola quality lines that have reduced glucosinolates in all parts of the plant. The *GTR*-edited lines also performed at par with the wild-type plants for their various seed-related parameters. It is proposed that the presence of a few functional *BjuGTR1* and *BjuGTR2* homologues, which were either non-targets or remained unedited in the *GTR*-edited lines, did not allow a complete blockage of the glucosinolate transport from and within the source tissues, which might otherwise affect the plant growth and developmental phenotypes as reported in the Arabidopsis *gtr1* and *gtr1gtr2* mutants (Kuo *et al.*, 2020; Nour-Eldin *et al.*, 2012; Saito *et al.*, 2015).

The strategy of simultaneous editing of both *GTR1* and *GTR2* homologues may also work in rapeseed where editing of only *GTR2* homologues has not provided the ideal low-SGC, high-LGC plants (He *et al.*, 2022; Tan *et al.*, 2022). The conventionally bred Canola quality lines of rapeseed have become increasingly susceptible to generalist pests in Europe and elsewhere leading to increased use of pesticides for crop protection (Zheng *et al.*, 2020). The recent discovery of glucosinolate exporters (UMAMIT29, UMAMIT30, and UMAMIT31) in Arabidopsis could provide new opportunities to improve the seed meal quality of the Brassica oilseed crops, without disturbing the distribution of defence compounds in the whole plant (Xu *et al.*, 2023).

We earlier developed mustard lines with low SGC using conventional breeding (Bisht *et al.*, 2009; Ramchiary *et al.*, 2007) and RNAi-suppression of *BjuMYB28* genes (Augustine *et al.*, 2013). However, the conventionally bred low SGC lines, due to reduced glucosinolate accumulation throughout the plant have been found to be vulnerable to heavy damage by termites, generalist pests, birds, and herbivores. The multiple GTR-edited lines reported in this study having Canola quality seed glucosinolate content and uncompromised defence and yield could prove to be a major breakthrough for mustard cultivation in the Indian subcontinent and elsewhere where mustard is a potential crop. Recently, India has adopted a conducive regulatory framework for the genome-edited crops belonging to the SDN-1 and SDN-2 categories. We could now carry out multisite trials of the transgene-free low-seed, high-leaf glucosinolate mustard lines developed in this study for their agronomical and defence performance. The study will contribute immensely towards developing ideal Canola quality lines with low SGC and high LGC in the related Brassica oilseeds, thus raising their oil- and seed-meal quality in the global market as well as reducing the dependency on agrochemicals in rapeseed and mustard cultivation.

Experimental procedures

Selection of gRNAs and generation of CRISPR/Cas9 construct

Full-length genomic sequences of *GTR1* (*BjuGTR1*) and *GTR2* (*BjuGTR2*) homologues from the *B. juncea* genome (Paritosh *et al.*, 2021) were used as queries to identify the 20 nt gRNAs using CRISPR-P v2.0 (http://crispr.hzau.edu.cn/CRISPR2/). Three gRNAs targeting a total of ten *BjuGTR* homologues were selected based on maximum on-score and minimum off-targets while containing 5′ G and 3′ NGG as PAM sequences for *Streptococcus pyogenes* Cas9. To generate the sgRNA cassette, the 20 nt seed sequence of gRNA was introduced between the AtU6-26 promoter and the scaffold in a two-step PCR reaction strategy, using customized PCR primers (Table S17). In the first step, two PCR reactions were performed to amplify the 'promoter-gRNA' and 'gRNA-scaffold' fragments, independently. In the second step, the two fragments were linked through another round of overlapping PCR to generate the AtU6-26 promoter:gRNA:scaffold expression cassette (Figure S2).

The *S. pyogenes* wild-type *Cas9* gene (*SpCas9*), driven by the constitutively expressing Cassava Mosaic Virus (CsVMV) promoter was cloned into the binary vector pZP200debar (Augustine *et al.*, 2013) containing the *bar* gene conferring resistance to herbicide 'Basta' (active ingredient phosphinothricin; Bayer, Germany) as a selectable marker to develop the pZP200debar::SpCas9 binary vector. All three sgRNA fragments were cloned at the appropriate restriction sites in the pZP200debar::SpCas9 vector to develop the GTR1:GTR2(GEd) construct.

Genetic transformation of *B. juncea* and development of *GTR*-edited lines

A high glucosinolate *B. juncea* variety—Varuna obtained from the Department of Genetics, UDSC, New Delhi had been maintained by selfing through several generations in the field. The 5-day-old hypocotyl explants were used to transform *B. juncea* using *Agrobacterium*-mediated genetic transformation, as per the protocol established in the laboratory (Augustine *et al.*, 2013). The T0 transformed plants were grown and maintained under contained net-house field conditions of NIPGR from November to April, following the guidelines laid by the Department of Biotechnology, Government of India. T0 transformants were confirmed through 'Basta' spray (200 mg/L). The confirmed T0 events were maintained by self-pollination to obtain the T1 and T2 seeds.

Isolation of genomic DNA and mutation screening

Genomic DNA was isolated from the young leaves for mutation analysis using the CTAB method (Murray and Thompson, 1980). Approximately 200 bp of the genomic sequence flanking the target site of each *BjuGTR* homologue was amplified using the homologue-specific primers (Table S17) and ExTaq DNA polymerase (TaKaRa Bio Inc.). PCR product was gel-eluted and sequenced by Sanger sequencing. The chromatogram file of each edited line was compared with that of the wild-type plant using the ICE analysis tool version 3.0 (Synthego Corporation) to identify the mutations in the target genes (Conant *et al.*, 2022). The percentage of plants showing editing of the target gene was used to calculate the editing frequency. Mutation efficiency for each gene was calculated based on the percentage of the cell population having insertions or deletions. The edited and wild-type sequences were aligned using different modules of the DNASTAR software (Lasergene).

Identification of transgene-free *GTR*-edited lines

Around 40–50 T1 seeds from each independent transformation event were grown in a containment net-house and checked for their sensitivity/resistance to herbicide 'Basta' (200 mg/L) by painting a few young leaves of 3–4-week-old plants with the herbicide. Segregation of T1 progeny for Basta-resistant (Cas9-containing) and sensitive (Cas9-free) phenotypes were recorded. Basta-sensitive transgene-free lines were confirmed for the

absence of the Cas9 and selectable marker (*bar*) genes by PCR analysis (Table S17).

Glucosinolate estimation

Total seed glucosinolate content (SGC) in seeds was estimated by the near-infrared reflectance spectroscopy in NIRS DS 2500 (FOSS, Denmark) using a calibrated equation (Gohain *et al*., 2021). The total and component glucosinolates from different tissue types namely seeds, leaves, roots, green pods, and developing seeds were analysed following an established HPLC-based protocol (Augustine *et al*., 2013; Gohain *et al*., 2021). Briefly, glucosinolates were extracted from 10 to 20 mg of the lyophilized tissue in 1 mL of 70% methanol containing the internal standard (50 μM sinalbin), the extract passed through a customized Sephadex-A column, and treated with sulphatase overnight. The desulpho-glucosinolates were eluted in 1 mL water and 10 μL run in a Shimadzu CLASS–VP V 6.14 HPLC machine on a reversed-phase C-18 column (Shimadzu Shim-pack GIST; 4.0 mm × 250 mm, 5 μm) using water (solvent A) and acetonitrile (solvent B) gradient (0.1–1 min, 1.5% B; 1–3 min, 1.5%–5% B; 3–5 min, 5%–7% B; 5–12 min, 7%–21% B; 12–15 min, 21%–29% B; 15–20 min, 29%–43% B; 20–24.50 min, 93% B and 24.50–30 min 1.5% B; flow rate of 1.0 mL/min), and peaks were detected and integrated at 229 nm using photodiode array detector. Glucosinolates concentration (μmoles/g DW) was determined by identifying the substrate peak of known glucosinolates and referencing it with the known internal standard peak (sinalbin) and applying the relative response factors (Burow *et al*., 2006). For each low SGC line, 8–10 independent T2 progeny (transgene-free) were analysed for determining the mean glucosinolate content and profiles in mature seeds, young leaf and root (1-month-old), flag leaf (at 50% flowering stage; ~2 months post sowing), developing green seeds, and silique walls (14–21 days post anthesis). One-way ANOVA was performed using Tukey's *post hoc* test in SPSS and $P < 0.05$ was considered significant. Graphs were plotted using GraphPad Prism 9.

Leaf sectioning of *B. juncea* plants

Fully expanded mature leaves from 2-months-old *B. juncea* plants grown in net-house were sectioned as described earlier (Kumar *et al*., 2017; Madsen *et al*., 2014). Briefly, the leaf in x-axis plane was dissected into margin (1 cm from the leaf outer margin), lamina (up to 2 cm from mid-vein), and mid-vein; while y-axis dissection separated the leaf tip (upper half), leaf base (lower half), and petiole. Similar-sized leaves from 6 independent T2 plants of each line were harvested in the morning and each leaf was subsectioned using a sharp scalpel, snap-frozen in liquid nitrogen, and lyophilized. Glucosinolate concentration was estimated using HPLC.

S. sclerotiorum detached leaf assays

The detached leaf assay was performed on *GTR*-edited lines, Canola quality doubled-haploid lines (DH-3325 and DH-2901; Ramchiary *et al*., 2007) and a BjuMYB28-RNAi line (C3/15; Augustine *et al*., 2013) along with wild-type Varuna plants as described previously (Kull *et al*., 2003). Similar-sized young leaves from 4-week-old plants grown in controlled growth chamber (Conviron, Canada) were harvested and infected with mycelial plugs of *S. sclerotiorum* strain SSD1 in square Petri plates. The detached leaves were inoculated at a fixed position in the leaf lamina with a 4 mm mycelial plug excised from the edge of an actively growing *S. sclerotiorum* culture over PDA. Infected leaves were incubated at 22 ± 2 °C under dark for 48 h and high >90% RH during infection, post which they were photographed to estimate the entire lesion areas (cm^2) using the ImageJ tool (1.52a, Rasband, 2018). Two independent experiments were conducted and the data was subjected to one-way ANOVA using SPSS and $P < 0.05$ was considered significant.

Feeding assay of *Spodoptera litura*

Spodoptera litura eggs obtained from the National Bureau of Agricultural Insect Resources (Bengaluru, India) were hatched on castor leaves, and subsequently, the larvae were maintained on an artificial diet at 22 ± 1 °C, 10 h light/14 h dark cycle and 60% RH. The second instar-staged larva was placed singly on a freshly detached young leaf (with an intact petiole) harvested from the 1-month-old plant and a no-choice feeding experiment was performed as described previously (Kumar *et al*., 2017). Larval weight gain was measured at 3, 6, and 9 days post-infestation (dpi), and the larval weight gain at 6 dpi and 9 dpi was estimated by subtracting the weight recorded at 3 dpi. Two independent experiments were conducted and the data were subjected to one-way ANOVA using Tukey's *post hoc* test in SPSS and $P < 0.05$ was considered significant. Graphs were plotted using GraphPad Prism 9.

Acknowledgements

This work was supported by the BT/HRD-NBA-NWB/38/2019-20 (10) grant of DBT (India) to N.C.B. Financial supports from CSIR (India) to A.M., UGC (India) to J.K., DBT (India) to R.K., and NIPGR Short-term fellowship to P.K. are acknowledged. Central facilities of DBT-NIPGR and technical help from Mr. Vinod Kumar, Mr. Haribhagwan Maurya, and field staff during the study are greatly acknowledged.

Conflict of interest statement

The authors declare that no conflict of interest exists.

Author contributions

NCB planned the research work. AM performed mutation screening in T0, T1, and T2 generations, glucosinolate estimations of seeds, leaves, roots, and silique walls of T1 and T2 generation plants and also helped in data analysis. JK developed editing constructs, generated *B. juncea* transformants, analysed seed glucosinolates in T0 and T1 plants, and carried out data analysis. RK developed the CRISPR/Cas9 binary vector, contributed to analysis, and discussed the results. PK performed the defence assays and assisted in leaf sectioning experiments. NCB, DP, and AKP collated the data, discussed the results, and drafted the article. All the authors read and approved the final article. NCB agrees to serve as the author responsible for contact and ensuring communication.

References

Andersen, T.G., Nour-Eldin, H.H., Fuller, V.L., Olsen, C.E., Burow, M. and Halkier, B.A. (2013) Integration of biosynthesis and long-distance transport establish organ-specific glucosinolate profiles in vegetative Arabidopsis. *Plant Cell*, **25**, 3133–3145.

Augustine, R., Mukhopadhyay, A. and Bisht, N.C. (2013) Targeted silencing of *BjMYB28* transcription factor gene directs development of low glucosinolate lines in oilseed *Brassica juncea*. *Plant Biotech. J.* **11**, 855–866.

Bell, L., Oloyede, O.O., Lignou, S., Wagstaff, C. and Methven, L. (2018) Taste and flavor perceptions of glucosinolates, isothiocyanates, and related compounds. *Mol. Nutr. Food Res.* **62**, 1700990.

Bisht, N.C., Gupta, V., Ramchiary, N., Sodhi, Y.S., Mukhopadhyay, A., Arumugam, N., Pental, D. *et al*. (2009) Fine mapping of loci involved with glucosinolate biosynthesis in oilseed mustard (*Brassica juncea*) using genomic information from allied species. *Theor. Appl. Genet.* **118**, 413–421.

Burow, M., Müller, R., Gershenzon, J. and Wittstock, U. (2006) Altered glucosinolate hydrolysis in genetically engineered *Arabidopsis thaliana* and its influence on the larval development of *Spodoptera littoralis*. *J. Chem. Ecol.* **32**, 2333–2349.

Burton, W.A., Ripley, V.L., Potts, D.A. and Salisbury, P.A. (2004) Assessment of genetic diversity in selected breeding lines and cultivars of canola quality *Brassica juncea* and their implications for canola breeding. *Euphytica*, **136**, 181–192.

Cartea, M.E. and Velasco, P. (2008) Glucosinolates in Brassica foods: bioavailability in food and significance for human health. *Phytochem. Rev.* **7**, 213–229.

Chen, S., Petersen, B.L., Olsen, C.E., Schulz, A. and Halkier, B.A. (2001) Long-distance phloem transport of glucosinolates in Arabidopsis. *Plant Physiol.* **127**, 194–201.

Cheng, F., Mandáková, T., Wu, J., Xie, Q., Lysak, M.A. and Wang, X. (2013) Deciphering the diploid ancestral genome of the mesohexaploid *Brassica rapa*. *Plant Cell*, **25**, 1541–1554.

Clay, N.K., Adio, A.M., Carine, C., Jander, G. and Ausubel, F.M. (2009) Glucosinolate metabolites required for an Arabidopsis innate immune response. *Science*, **323**, 95–101.

Conant, D., Hsiau, T., Rossi, N., Oki, J., Maures, T., Waite, K., Yang, J. *et al*. (2022) Inference of CRISPR edits from Sanger trace data. *CRISPR J.* **5**, 123–130.

van Dam, N.M., Tytgat, T.O.G. and Kirkegaard, J.A. (2009) Root and shoot glucosinolates: a comparison of their diversity, function and interactions in natural and managed ecosystems. *Phytochem. Rev.* **8**, 171–186.

Gatrell, S., Lum, K., Kim, J. and Lei, X.G. (2014) Nonruminant nutrition symposium: potential of defatted microalgae from the biofuel industry as an ingredient to replace corn and soybean meal in swine and poultry diets. *J. Anim. Sci.* **92**, 1306–1314.

Gohain, B., Kumar, P., Malhotra, B., Augustine, R., Pradhan, A.K. and Bisht, N.C. (2021) A comprehensive Vis-NIRS equation for rapid quantification of seed glucosinolate content and composition across diverse Brassica oilseed chemotypes. *Food Chem.* **354**, 129527.

Halkier, B.A. and Gershenzon, J. (2006) Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol.* **57**, 303–333.

He, Y., Yang, Z., Tang, M., Yang, Q.Y., Zhang, Y. and Liu, S. (2022) Enhancing canola breeding by editing a glucosinolate transporter gene lacking natural variation. *Plant Physiol.* **188**, 1848–1851.

Hopkins, R.J., van Dam, N.M. and van Loon, J.J.A. (2009) Role of glucosinolates in insect plant relationships and multitrophic interactions. *Annu. Rev. Entomol.* **54**, 57–83.

Insecticide Resistance Action Group. (2019) *Insecticide Resistance status in UK oilseed rape crop*. https://ahdb.org.uk/knowledge-library/the-insecticide-resistance-action-group-irag (accessed May 27, 2023).

Jørgensen, M.E., Nour-Eldin, H.H. and Halkier, B.A. (2015) Transport of defense compounds from source to sink: lessons learned from glucosinolates. *Trends Plant Sci.* **20**, 508–514.

Kull, L.S., Vuong, T.D., Powers, K.S., Eskridge, K.M., Steadman, J.R. and Hartman, G.L. (2003) Evaluation of resistance screening methods for *Sclerotinia* stem rot of soybean and dry bean. *Plant Dis.* **87**, 1471–1476.

Kumar, P., Augustine, R., Singh, A.K. and Bisht, N.C. (2017) Feeding behaviour of generalist pests on *Brassica juncea*: implication for manipulation of glucosinolate biosynthesis pathway for enhanced resistance. *Plant Cell Environ.* **40**, 2109–2120.

Kuo, H.Y., Kang, F.C. and Wang, Y.Y. (2020) Glucosinolate Transporter1 involves in salt-induced jasmonate signaling and alleviates the repression of lateral root growth by salt in Arabidopsis. *Plant Sci.* **297**, 110487.

van Leur, H., Vet, L.E., van der Putten, W.H. and van Dam, N.M. (2008) Barbarea vulgaris glucosinolate phenotypes differentially affect performance and preference of two different species of lepidopteran herbivores. *J. Chem. Ecol.* **34**, 121–131.

Love, H.K., Rakow, G., Raney, J.P. and Downey, R.K. (1990) Development of low glucosinolate mustard. *Can. J. Plant Sci.* **70**, 419–424.

Lysak, M.A., Cheung, K., Kitschke, M. and Bures, P. (2007) Ancestral chromosomal blocks are triplicated in Brassiceae species with varying chromosome number and genome size. *Plant Physiol.* **145**, 402–410.

Madsen, S.R., Olsen, C.E., Nour-Eldin, H.H. and Halkier, B.A. (2014) Elucidating the role of transport processes in leaf glucosinolate distribution. *Plant Physiol.* **166**, 1450–1462.

Milovac, Ž., Zorić, M., Franeta, F., Terzić, S., Petrović Obradović, O. and Marjanović, J.A. (2017) Analysis of oilseed rape stem weevil chemical control using a damage rating scale. *Pest Manag. Sci.* **73**, 1962–1971.

Murray, M.G. and Thompson, W.F. (1980) Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **8**, 4321–4325.

Nambiar, D.M., Kumari, J., Augustine, R., Kumar, P., Bajpai, P.K. and Bisht, N.C. (2021) GTR1 and GTR2 transporters differentially regulate tissue-specific glucosinolate contents and defence responses in the oilseed crop *Brassica juncea*. *Plant Cell Environ.* **44**, 2729–2743.

Nour-Eldin, H.H., Andersen, T.G., Burow, M., Madsen, S.R., Jørgensen, M.E., Olsen, C.E., Dreyer, I. *et al*. (2012) NRT/PTR transporters are essential for translocation of glucosinolate defence compounds to seeds. *Nature*, **488**, 531–534.

Nour-Eldin, H.H., Madsen, S.R., Engelen, S., Jørgensen, M.E., Olsen, C.E., Andersen, J.S., Seynnaeve, D. *et al*. (2017) Reduction of antinutritional glucosinolates in Brassica oilseeds by mutation of genes encoding transporters. *Nat. Biotechnol.* **35**, 377–382.

Paritosh, K., Yadava, S.K., Singh, P., Bhayana, L., Mukhopadhyay, A., Gupta, V., Bisht, N.C. *et al*. (2021) A chromosome-scale assembly of allotetraploid *Brassica juncea* (AABB) elucidates comparative architecture of the A and B genomes. *Plant Biotechnol. J.* **9**, 602–614.

Potts, D., Rakow, G. and Males, D.R. (1999) *Canola quality Brassica juncea*, a new oilseed crop for the Canadian prairies. *Proceedings 10th Int. Rapeseed Congress, Canberra, Australia, 26–29 September (CD-ROM)*.

Ramchiary, N., Bisht, N.C., Gupta, V., Mukopadhyay, A., Arumugam, N., Sodhi, Y.S. *et al*. (2007) QTL analysis reveals context-dependent loci for seed glucosinolate trait in oilseed *Brassica juncea*: Importance of recurrent selection backcross scheme for the identification of 'true' QTL. *Theor. Appl. Genet.* **116**, 77–85.

Rasband, W.S. (2018) *ImageJ*. Bethesda, Maryland: National Institutes of Health.

Raymer, P.L. (2002) Canola: an emerging oilseed crop. *Trends New Crops New Uses*, **1**, 122–126.

Saito, H., Oikawa, T., Hamamoto, S., Ishimaru, Y., Kanamori-Sato, M., Sasaki-Sekimoto, Y., Utsumi, T. *et al*. (2015) The jasmonate-responsive GTR1 transporter is required for gibberellin-mediated stamen development in *Arabidopsis*. *Nat. Commun.* **6**, 6095.

Sekulic, G. and Rempel, C.B. (2016) Evaluating the role of seed treatments in canola/oilseed rape production: integrated pest management, pollinator health, and biodiversity. *Plan. Theory*, **5**, 32.

So, K.K.Y. and Duncan, R.W. (2021) Breeding Canola (*Brassica napus* L.) for protein in feed and food. *Plants (Basel)*, **10**, 2220.

Tan, Z., Xie, Z., Dai, L., Zhang, Y., Zhao, H., Tang, S., Wan, L. *et al*. (2022) Genome- and transcriptome-wide association studies reveal the genetic basis and the breeding history of seed glucosinolate content in Brassica napus. *Plant Biotechnol. J.* **20**, 211–225.

Wolt, J.D. and Wolf, C. (2018) Policy and governance perspectives for regulation of genome-edited crops in the United States. *Front. Plant Sci.* **9**, 1606.

Xu, D., Hanschen, F.S., Witzel, K., Nintemann, S.J., Nour-Eldin, H.H., Schreiner, M. and Halkier, B.A. (2017) Rhizosecretion of stele-synthesized glucosinolates and their catabolites requires GTR-mediated import in Arabidopsis. *J. Exp. Bot.* **68**, 3205–3214.

Xu, D., Hunziker, P., Koroleva, O., Blennow, A., Crocoll, C., Schulz, A., Nour-Eldin, H.H. *et al*. (2019) GTR-Mediated radial import directs accumulation of defensive glucosinolates to sulfur-rich cells in the

phloem cap of Arabidopsis inflorescence stem. *Mol. Plant*, **12**, 1474–1484.

Xu, D., Sanden, N.C.H., Hansen, L.L., Belew, Z.M., Madsen, S.R., Meyer, L., Jørgensen, M.E. *et al*. (2023) Export of defensive glucosinolates is key for their accumulation in seeds. *Nature*, **617**, 132–138.

Zheng, X., Koopmann, B., Ulber, B. and von Tiedemann, A. (2020) A global survey on diseases and pests in oilseed rape – Current challenges and innovative strategies of control. *Front. Agronomy*, **2**, 590908.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Guide RNA conserved target sites in *B. juncea GTR1* and *GTR2* homologues.
Figure S2 Generation of sgRNA through an overlapping PCR.
Figure S3 Summary of the CRISPR/Cas9-induced mutations observed in the 17 low SGC GTR-edited T0 events.
Figure S4 Identification of transgene-free *GTR*-edited lines in the T1 generation.
Table S1 Estimation of fractions and total SGC (μmoles/g DW) in the T1 seeds of BjuGTR-edited events and the WT Varuna plants.
Table S2 Analysis of CRISPR/Cas9-induced mutation in the *BjuGTR* homologues in 22 selected BjuGTR-edited lines in the T0 generation.
Table S3 Summary of Cas9-free (transgene-free) T1 plants obtained for each transformation event derived from GTR1: GTR2(GEd) construct.
Table S4 Estimation of total glucosinolate content and fractions (μmoles/g DW) in T2 seeds of *GTR*-edited lines and the WT Varuna plants.
Table S5 Estimation of total glucosinolate content and fractions (μmoles/g DW) in mature T3 seeds (SGC) of 14 transgene-free low SGC lines and WT Varuna plants.
Table S6 Analysis of CRISPR/Cas9-induced mutations in the *BjuGTR* homologues in the T2 generation of the selected transgene-free *GTR*-edited lines.
Table S7 Estimation of total glucosinolate content and fractions (μmoles/g DW) in 1-month-old T2 leaf (LGC) of 14 transgene-free low SGC lines and WT Varuna plants.
Table S8 Estimation of total glucosinolate content and fractions (μmoles/g DW) in 1-month-old T2 root (RGC) of 14 transgene-free low SGC lines and WT Varuna plants.
Table S9 Estimation of total glucosinolate content and fractions (μmoles/g DW) in T2 flag leaf (LGC) of 14 transgene-free low SGC lines and WT Varuna plants.
Table S10 Estimation of total glucosinolate content and fractions (μmoles/g DW) in the silique walls (SWGC) of 14 transgene-free low SGC lines and the WT Varuna plants.
Table S11 Estimation of total glucosinolate content and fractions (μmoles/g DW) in the developing green seeds (gSGC) of 14 transgene-free low SGC lines and the WT Varuna plants.
Table S12 Estimation of total glucosinolate content and fractions (μmoles/g DW) in the leaf sections (*x*-axis) of eight transgene-free low SGC lines and the WT Varuna plants.
Table S13 Estimation of total glucosinolate content and fractions (μmoles/g DW) in the leaf sections (*y*-axis) of the eight transgene-free low SGC lines and the WT Varuna plants.
Table S14 Lesion area of *S. sclerotiorum* infection in detached leaf assays on *GTR*-edited lines at 48 hpi.
Table S15 Weight gain of *Spodoptera litura* infection in detached leaf assays on GTR-edited lines at 6 dpi and 9 dpi.
Table S16 Seed quality and yield-related parameters in mature T3 seeds of 14 low SGC lines and WT Varuna seeds estimated using near-infrared spectroscopy.
Table S17 List of primers used in this study.