Int.J.Curr.Microbiol.App.Sci (2018) *7*(12): 3346-3357

International Journal of Current Microbiology and Applied Sciences
***ISSN: 2319-7706* Volume 7 Number 12 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.712.385

Variability in Total Glucosinolate Content in F_2 Generation of *B. juncea* and their Molecular Validation

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Keywords

Brassica juncea, Total glucosinolate, Molecular markers, F_2 populations

Article Info

Accepted:
24 November 2018
Available Online:
10 December 2018

A B S T R A C T

To increase the utilization of seed meal and to improve the feed palatability it is necessary to develop low glucosinolate mustard lines. The present study was intended to develop low glucosinolate varieties in *B.juncea* through evaluation of variability in total glucosinolate among parental and F_2 population. Crosses between NRCHB101×PDZ-1, NRCHB101×RLC-3, NRCHB101×HEERA, DRMR150-35×PDZ-1, DRMR150-35×RLC-3 and NRCHB101×HEERA generated 252 F_2 populations. These F_2 populations showed the frequency of distribution of total glucosinolate to range from18 µmol/g to 168 µmol/g which is out of the range of parental lines. Clustering of all the progenies derived from the crosses showed considerable diversity in their total glucosinolate content. 8 Lines derived from crosses DRMR150-35×PDZ-1 and NRCHB101× RLC-3 having total glucosinolate content less than 30 µmol/g were reconfirmed by molecular markers.

Introduction

Oilseed brassica with its history of origination from central Asia particularly from India could be an ideal source to tap in various traits required for crop improvement. One of the most sought after traits for quality improvement is low glucosinolate content. Members of Brassicaceae family are rich sources of sulfur and nitrogen containing compounds collectively known as Glucosinolates. Their diverse structures contribute to diversity in functions in plant system. Its presence adds to the unique odour, pungency and flavour of mustard oil. The pungency of mustard oil is preferred among majority of the Indian population as it adds taste to many indigenous cuisines. The presence of enzyme myrosinase hydrolyse the intact glucosinolate upon plant wounding releasing their products which have beneficial as well as harmful effects to consumers (Fahey *et al.*, 2001;Wittstock *et al.*, 2002). One of the breakdown products is the allylisothiocyanates which adds to the pungency. The seed meal remaining after oil extraction in case of Indian mustardusually contains a high amount of glucosinolates (80-160 µmol/g), which reduces its value as livestock feed. At the same time the breakdown products of glucosinolates have been reported to have anti-carcinogenic

properties as well (Juge *et al.*, 2007; Cartea and Velasco 2008; Traka and Mithen, 2009) and vital role in defence against pests and pathogens (Clay *et al.*, 2009; Hopkins *et al.*, 2009).

To increase the utilization of seed meal and to improve the feed palatability it is necessary to develop low glucosinolate mustard lines. The present study was intended to develop low glucosinolate varieties in *B.juncea* through evaluation of variability in total glucosinolate among parental and F_2 population. The presence of more than 120 types of glucosinolates suggests the complex regulation of its pathway. As many researcher like Halkier and Gershenzon (2006) have reported that a network of genes are involved in genetically controlling glucosinolate content and its quantitative inheritance is basically regulated by complex genetic factors depending on environmental conditions (Hirani *et al.*, 2012), variety, age, growing season and plant parts (Zhang *et al.*, 2008).Molecular markers linked to glucosinolate trait were also validated in lines (F_2 population) having glucosinolate content less than 30µmol/g.

Materials and Methods

Experimental site

Experiment was initiated during 2016-17 at the experimental area of ICAR-Directorate of Rapeseed-mustard, Bharatpur, India. Experimental area having sandy loam soil with pH 8.5, geographically situated at 27°11'N Lat. and 77°27'E Long, and 180 m above sea level. Overall it was good monsoon year with 765.2 mm rainfall in 41 rainy days. The monsoon withdrew in last week of September leaving sufficient conserved moisture for subsequent mustard crop. Throughout the cropping season the mean monthly temperature remained favourable for rapeseed mustard production. However daily maximum temperature reached around 4.8°C during last week of Dec. 2016. The average sunshine during crop season was 3.8-10.3 hours.

Experimental design

F_1 plants and their parents that is NRCHB101, DRMR150-35, PDZ-1, RLC-3 and Heera (Table 1) were sown (Rabi season-2016-17) in 5 rows plot of 5 m length spaced 30 cm apart with plant to plant spacing of 10 cm followed by thinning after every 15-20 days. Recommended package of practices for raising a healthy crop was followed. F_2 seed samples derived from six crosses [NRCHB101×PDZ-1, NRCHB101×RLC-3, NRCHB101×HEERA, DRMR150-35×PDZ-1, DRMR150-35×RLC-3 and NRCHB101×HEERA] involving low glucosinolate varieties as donors and high yielding varieties as recipients (Table 2) were harvested at maturity. The seeds were collected in paper bags and were stored at room temperature until further biochemical and molecular analysis.

Crossing and selection scheme

Two high glucosinolate cultivars of *B. juncea* that is NRCHB101 and DRMR150-35 were used as female and were crossed with low glucosinolate *B. juncea* cultivars that are PDZ-1, RLC-3 and Heera (Table 2). Emasculation of maternal flower buds was performed 24–48 h before anthesis, and fresh pollen from the paternal parent was applied to the stigmas. The flowers were then protected with paper bags and labeled. The resultant F_1 plants selfed to produce the F_2 generation. Total glucose inolate content of seeds derived from individual plants were analysed for glucosinolate and molecular validation as described below. A negative selection based on visual observations was conducted among

all the six F_2plant generation in order to eliminate plants or lines resembling morphological characteristics with any of the *B. juncea* parents that is NRCHB101, DRMR150-35, PDZ-1, RLC-3 and HEERA. However, intermediate types were selected further. A total of 27, 66, 48, 37, 54, 18 F_2 plants were selected from F_2 crosses NRCHB101×PDZ-1, NRCHB101×RLC-3, NRCHB101×HEERA, DRMR150-35×PDZ-1, DRMR150-35×RLC-3, NRCHB101×HEERA respectively (Table 2) for a detailed characterisation at phenotypic and molecular level they were selected from an initial population of 90, 85, 92, 80, 101, 54 F2 plants with respect to crosses J1, J2, J3, J4, J5 and J6 respectively (The letter "J" representing species *juncea*).

Seed glucosinolate extraction and quantification

Total glucosinolate was estimated in seeds of 252 F_2plantsaccording to the method standardized by Mawlong *et al.,* (2017). The ground seeds were defatted by homogenizing with n-hexane 3-4 times until oil was completely removed. Defatted seed meal was allowed to dry and then used for total glucosinolate estimation. The complex formation between glucosinolates methanolic extract and palladium was estimated by taking the absorbance at 425nm using a spectrophotometer (Labomed UV-VIS Double beam UVD-3500).

The absorbance obtained was used to calculate total glucosinolate content using the formula; Total glucosinolate conc. (µmol/g) = 1.40 + 118.86 × A_{425}**,** Where, A_{425}= absorbance at 425. All analysis were done side by side with two national checks already reported for high and low glucosinolates - Varuna and Hayola(All Indian Coordinated Research Project-Rapeseed Mustard, 2017) for accuracy.

DNA extraction and PCR analysis

Genomic DNA from matured seeds was isolated using the standard cetyl trimethyl ammonium bromide (CTAB) protocol (Murray and Thompson, 1980 and Doyle and Doyle, 1990). A set of five polymorphic primers i.e. (GER-1MRPR + IP3GER-1F(Q1); Myb28(Q2); At5g41(Q3); At5GAJ67(Q4) and GER-5FPF+GER-5MRPR(Q5) (Bisht *et al.*, 2009) (Table 3) were used for amplification of parents and F_2 populations derived from crosses J1, J2, J3, J4, J5 and J6.PCR amplification was carried out in a total volume of 10 µl containing 25ng of genomic DNA, 1.0 unit DNA polymerase (Dream Taq),10X PCR assay buffer with 1.5mM $MgCl_2$, 20 ng each primer and 0.2 µl dNTPs mix. The volume was made up to 10 µl using nuclease free water.

Amplification was carried out in96-well Fast Thermal Cycler (PE Applied Biosystems, USA). With initial denaturation at 94°C for 5 min, cyclic denaturation at 94°C for 45 s, annealing temperature at 55-58°C for 50 s and the primer extension at 72°C for 1min and final extension of 72°C for 7 min.. The cycle was repeated 35 times followed by incubation at 4°C. PCR-amplified products were electrophoretically separated on 2.5% agarose gel containing 0.01% ethidium bromide prepared in 1xTAE (Tris-Acetic acid-EDTA).

Amplicon sizes were predicted by comparing with 100bp DNA Ladder (Thermo Scientific). The gel was run for 3 h at 80V. After electrophoresis, the amplification products were visualized in a gel documentation system (IG/LHR, Syngene, UK).

Statistical analysis

Cluster analysis was done using SAS software 9.4 available at ICAR-Indian agricultural statistical research institute, New Delhi

Results and Discussion

Variation in total glucosinolate content in F_2 generation

With the aim to validate the F_2 population for segregation of quality trait total glucosinolate content was estimated. The F_2 plants exhibited wide range of total glucosinolate content from18.52 to168.60 µmol/g, with mean of 67.62±1.67 µmol/g seed (Table 2). As observed from the distribution frequency plot (Fig. 1) maximum F_2 populations showed glucosinolate content in a range of 61 to 80 µmol/g (77). This was followed by 67 F_2 populations in a range of 41-60 µmol/g seed and 41 F_2populations inarange of 81-100 µmol/g seed. Range of 21-40 µmol/g and 101-120µmol/g was showed by 38 and 17 numbers of samples respectively and it was found that there were only 2 F_2 populations in 0-20 µmol/g seed. Kumar *et al.,* (2004) had put an insight into seed glucosinolate estimation and had reported the range of 85-250 µmol/g glucosinolate content in defatted seed meal. However, wide range of total glucosinolates content was reported by many researchers from various studies done earlier (Padilla *et al.,* 2007, Bellostas *et al.,* 2007, Verkerk *et al.,* 2009 and Yang and Quiros 2010). Amongst the selected F_2 plants, a total of 8 plants derived from crosses DRMR150-35×PDZ-1 and NRCHB101× RLC-3 were found to be highly promising in terms of low glucosinolate content (<30µmol/g defatted seed meal). However, moderately low glucosinolate (<60 µmol/g defatted seed meal) was found in plants obtained from crosses DRMR150-35×RLC-3, DRMR150-35×PDZ-1, NRCHB101×HEERA, NRCHB101×RLC-3 and NRCHB101× HEERA in 10, 26, 14, 46, 6plants respectively. In the present study the total glucosinolate content ofF_2 segregants exceeded the parental range of NRCHB-101, DRMR-150-35, PDZ-1, RLC-3 and HEERA which were reported to be 115.56 µmol/g, 90.55 µmol/g, 19.96 µmol/g, 14.07 µmol/g and 33.19 µmol/g of seed respectively (Table 2). This resulted due to transgressive segregants in both the directions for low as well as high glucosinolates as seen from the frequency distribution graph (Fig. 1) which explains that there is gene interaction and recovery of transgressive segregants for both high and low glucosinolates. This also confirms that total glucosinolates content is a quantitative trait. The analysis of total glucosinolate content in the present study among different accessions of *B.juncea* and their F_2 generations indicate that there is scope for additional reduction of glucosinolate levels so as to achieve the Canola criteria for Indian mustard.

Cluster analysis

To understand the diversification we clustered the progenies derived from six parental crosses. The six clusters labeled as C-J1, C-J2, C-J3, C-J4, C-J5 and C-J6(where 'C' represents cluster and J1, J2, J3, J4, J5 and J6 stands for respective crosses) were formed, one for each F_2 population (Fig. 2) The six clusters revealed distinct variability between F_2 populations for glucosinolate content. The F_2 population derived from crossesNRCHB101×RLC-3 and DRMR150-35×PDZ-1 shows maximum variation in glucosinolate content ranging from below 30 to more than 100µmol/g of defatted seed meal (Table 2).

In cluster C-J1 a cross between NRCHB 101× PDZ 1 generates 27 lines, out of which two major clusters were obtained using the SAS software with 4 lines lying within C-I and the rest within C-II. Looking into their total glucosinolate content it was observed that F_2 populations less than 70 µmol/g were clustered in C-II whereas, glucosinolate content above 100µmol/g were grouped in another cluster C-I (Fig. 2a).

In case of CJ2, a cross between NRCHB 101 × RLC-3 cluster analysis showed two major groups C-I with 22 lines and C-II with 46 lines. Based on the glucosinolate content C-II was subdivided into two subgroup C-II a (18 lines) and C-II b (28 lines). Narrowing down the C- II b we further observed that it can be subdivided into two subclade CII b(i) (23lines) and CII b(ii) (5 lines). Looking back into the range of glucosinolate contents, we observed subclade CII b(ii) to have lines with less than 35µmol/g, while the rest of the clusters have total glucosinolate content more than 100µmole/g (Fig. 2b). Coming to the third cross between NRCHB 101 × Heera (Fig. 2c) a total of 48 progenies were obtained again based on their spectral range of glucosinolate content. We clusterd them into two major groups C-I (6) and C-II (42).

The 6lines of C-I were found to contain total glucosinolate content of more than 100µmole/g while, the rest 43 lines were within the range of 31.62 to 91.39 µmol/g, based on this we further sub grouped C-II into IIa (26 lines) and IIb (16 lines), these 16 lines were further subdivided into subclade C-IIb(i) (4lines) and C-II b(ii) (12lines). Among the two subclade, C-IIb(i) contains total glucosinolate content less than 35µmol/g.

Table.1 Parental genotypes used for F_2 generation and glucosinolate analysis along with their pedigree

S. No.	Genotype	Species	Pedigree	Country/Developing institute
1.	NRCHB-101	*Brassica juncea*	BL4×Pusa bold	ICAR-DRMR, Bharatpur
2.	DRMR150-35	*Brassica juncea*	RH519×Pusa bold	ICAR-DRMR, Bharatpur
3.	PDZ-1	*Brassica juncea*	LES-1-27/NUDHYJ-3	IARI, New Delhi
4.	RLC-3	*Brassica juncea*	JM 06003/JM 06020	PAU, Ludhiana
5.	HEERA	*Brassica juncea*	ZYR-4/BJ-1058	Nagpur University & Dhara Veg. Oil & Food Co. Ltd., Vadodara

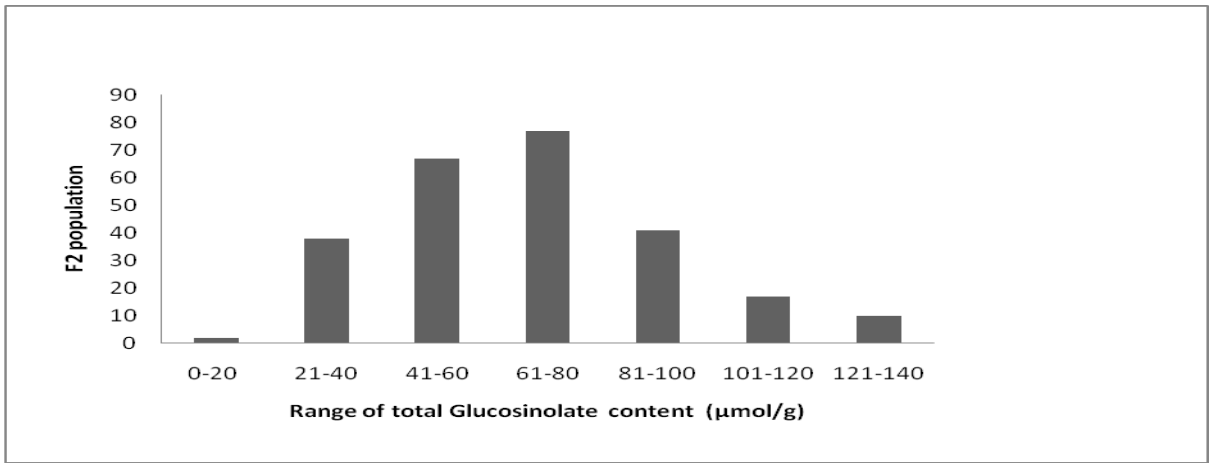
Table2. Mean and standard error for glucosinolate concentrations in μmol/g of seed, and number of plants analysed in parental, F_2 generation

Generations	Total Glucosinolate content (µmol g^{-1}seed meal)			No. of plants Assayed
	RANGE	Mean ±SEM	Standard deviation	
Parents				
NRCHB101	113.98-116.52	115.56±0.24	0.78	10
DRMR150-35	89.13-91.56	90.55±0.24	0.75	10
PDZ-1	18.82-20.79	19.96±0.18	0.58	10
RLC-3	12.92-15.03	14.07±0.19	0.61	10
HEERA	32.9-34.09	33.19±0.22	0.71	10
F2 PLANTS				
NRCHB101×PDZ-1(J1)	51.59-109.19	70.32 ±2.97	15.48	27
NRCHB101×RLC-3(J2)	18.52-113.64	53.26 ±2.60	21.82	66
NRCHB101×Heera(J3)	31.62-128.55	70.94 ±2.95	20.90	48
DRMR150-35×PDZ-1(J4)	25.37-73.83	49.33 ±2.10	13.13	37
DRMR150-35×RLC-3(J5)	35.83-168.80	84.76 ±3.67	27.49	54
DRMR150-35 × Heera(J6)	62.65-141.42	95.19 ±4.50	20.18	18

Table.3 Details of glucosinolate QTLs linked markers used for validation in parent and F2 progenies. (Pushpa *et al.*, 2016; Bisht *et al.*, 2009)

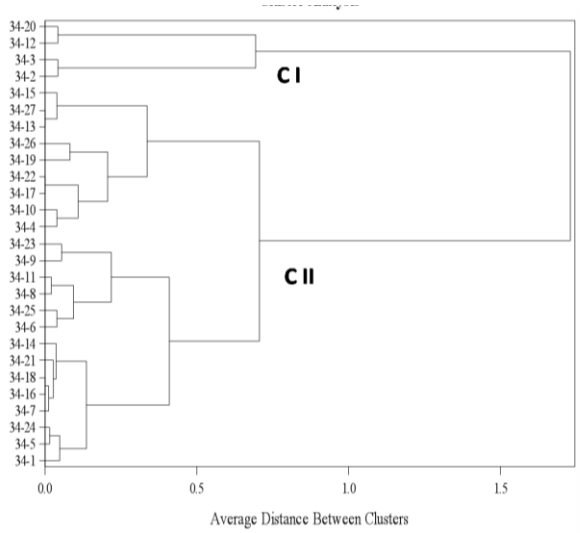
Primer Code	Primer Name	Nucleotide sequence (5′→3')	QTL Name	Linkage group	Co-localised candidate gene	PCR Product	Tm (^{0}C)
Q1	GER-1MRPR	5’CATGTCGACGACTTCTTCTCTAGTCA	*J2Gsl1*	A2	*BjuA.GSL-ELONG.a*	950(H)/650 (L) bp	55^{0}C
	IP3GER-1 F	5’GGTTTTCCCTTGGATTTGAGTCT					
Q2	Myb28R	5’TATCCTCTTCATTGACAATCTGCTCAG	*J17Gsl5*	A3	*BjuA.Myb28.a BjuA.GSL-ELONG.c,d*	1020(H)/1000 (L)bp	58^{0}C
	Myb28F	5’AAGGGGCATGGACCACCGA					
Q3	At5g41F	5’GTTTCAGGGTGACTCTCCTCTTG	*J9Gsl3*	A9	---	800(H)/770 (L)bp	58^{0}C
	At5g41R	5’GCTTGTGCACCTTCATCGTC					
Q4	At5GAJ67F	5’CAGCGTAAGGAAGAAGAGAGAC	*J9Gsl3*	A9	---	450(H)/- bp	58^{0}C
	At5GAJ67FR	5’CCATCACTATGTCATTTGCCA					
Q5	GER-5FPF	5’GACATCATGGAAGTTGGTTTCCCTGC	*J3Gsl2*	A3	*BjuA.GSL-ALK.a*	350(H)/310(L) bp	55^{0}C
	GER-5MRPR	5’GGGAGGTAACCTGTTTCTTCATCCA					

Fig.1 Graphical presentation showing frequency distribution of total glucosinolates content in F_2 populations of *B.juncea*

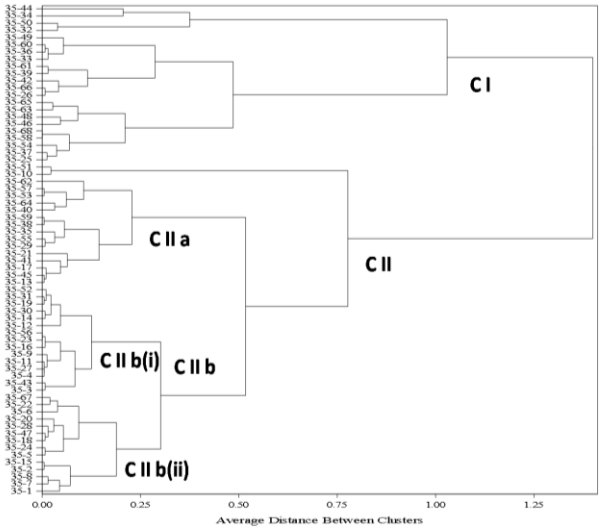


Range of Total Glucosinolate (µmol/g)	No. of F_2 samples
0-20	2
21-40	38
41-60	67
61-80	77
81-100	41
101-120	17
121-140	10

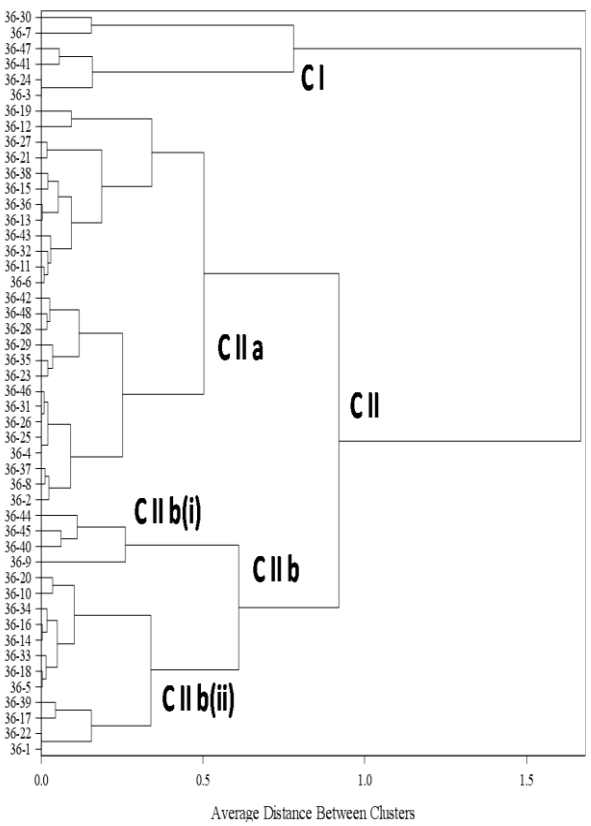
Fig.2 The progenies derived from each cross of (a) NRCHB 101× PDZ-1 (CJ1), (b) NRCHB 101× RLC3 (CJ2), (c) NRCHB 101× Heera (CJ 3),(d) DRMR 150-35 × PDZ-1 (CJ4), (e) DRMR 150-35 × RLC-3 (CJ5) and (f) DRMR 150-35 × Heera (CJ6) were clusterd using the SAS software based on total glucosinolate content



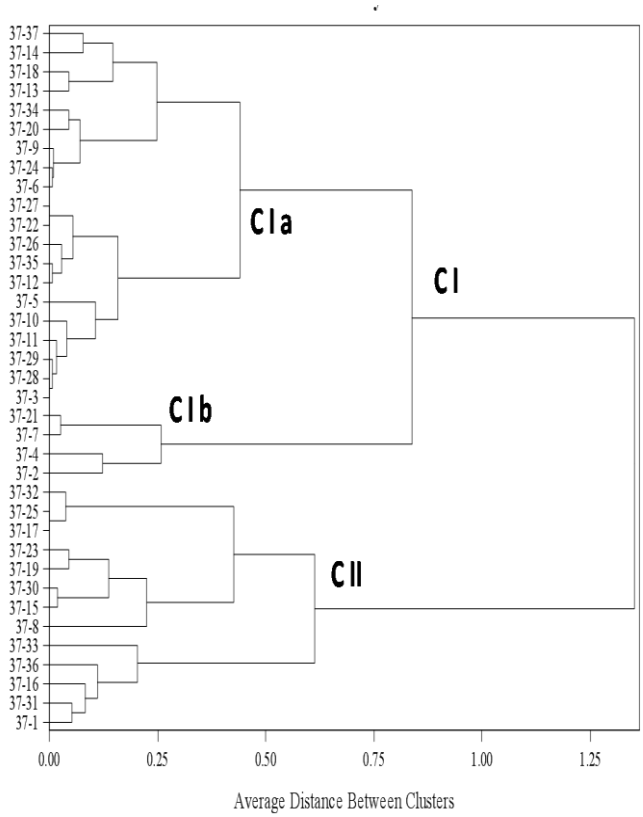
(a) CJ1



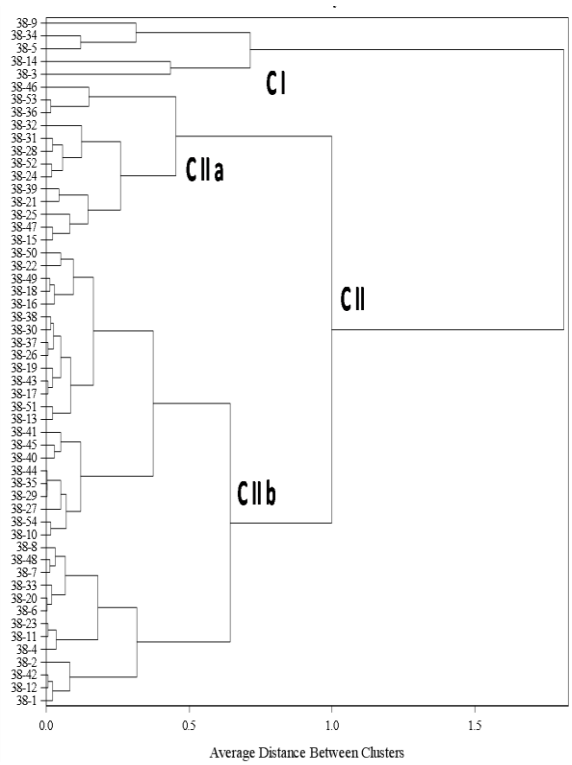
(b) CJ2



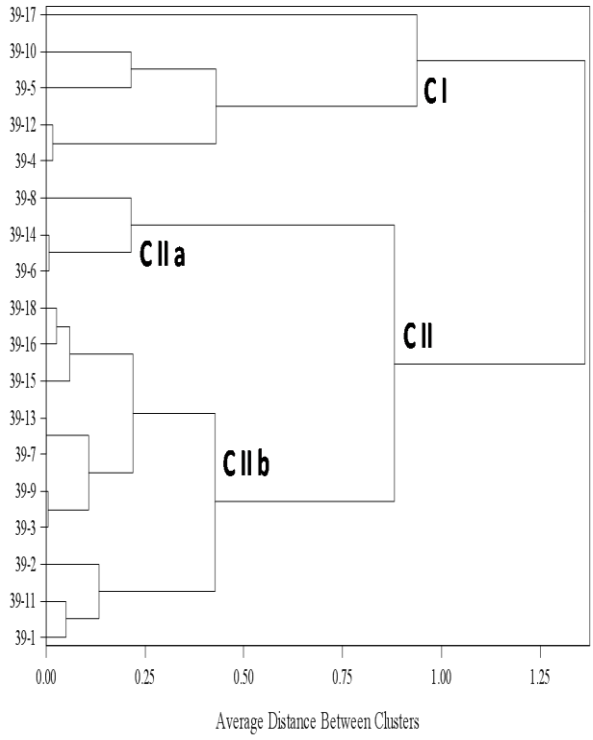
(c) CJ3



(d) CJ4

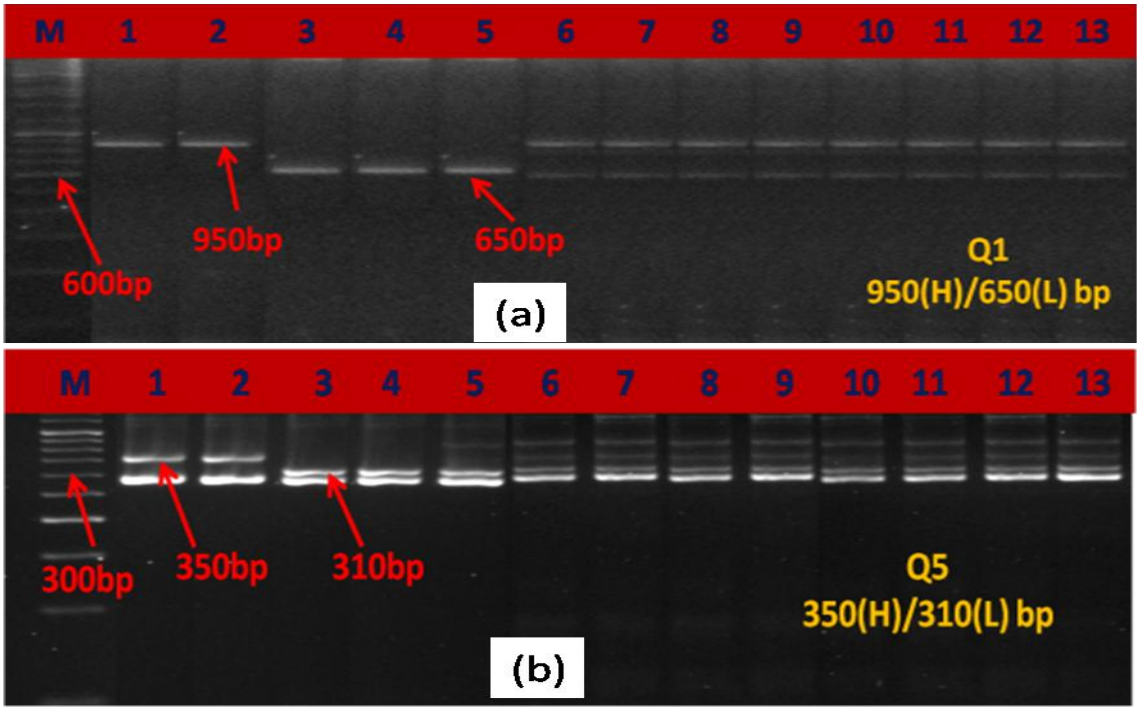


(e) CJ5



(f) CJ6

Fig.3 Polymerase chain reaction amplification products from *B.juncea* genotypes and F_2 generation using marker markers linked to quality (Glucosinolate); (a) Represent bands amplified with marker GER-1MRPR+ IP3GER-1F (Q1), (b)Represent bands amplified with marker GER-5FPF + GER-5MRPR (Q5).The representation of each lanes are as follows: *lane* 'M' represent 100bp DNA marker; *lane*1NRCHB101; *lane*2-DRMR150-35; *lane*3PDZ-1; *lane*4RLC-3; *lane*5Heera; *lane*6 and7 progenies derived from NRCHB 101×RLC-3; *lane*8 to 13 progenies derived from DRMR150-35 × PDZ-1



Cluster CJ4 a cross between DRMR 150-35 × PDZ-1 gave 37 progenies which were clustered into two major groups C-I (24 lines) and C-II (13 lines).

The C-I with 24 lines were subdivided into C-I a (20lines) and C-I b (4lines). Out of which C I b is reported to contain total glucosinolate less than35µmol/g (Fig. 2d).

Cluster CJ5 across between DRMR 150-35× RLC-3 gave 54 progenies which were clustered into two major groups C-I (5 lines) and C-II (49 lines) It was observed that all the 5 lines of C-I were reported to have total glucosinolate content of more than 100 µmol/g while, were within a range of 35.83 to 112.32 µmol/g.

A cross between DRMR150-35 × Heera gave 18 progenies which were classified into two clusters C-I (5) and C-II (13) of which none were having total glucosinolate content less than 35µmol/g,it ranged between 62.65 and 141.42 µmol/g.

Clustering of each crosses simplifies the process of selection of potential lines for the next stage in plant breeding. The variation in total glucosinolates from each cross hints the epistatic interaction of genes involved in the glucosinolate pathway.

Molecular characterization

To validate the potential lines from each crosses and to reconfirm the segregating

progenies, molecular markers that were already reported (Bisht *et al.,* 2009) (Table 3) were used for both parents and selected 8 F_2 progenies derived between DRMR150-35×PDZ-1 and NRCHB101× RLC-3 having total glucosinolate content less than 30µmol/g.A set of five primers i.e. (GER-1MRPR + IP3GER-1F(Q1); Myb28 (Q2); At5g41 (Q3); At5GAJ67 (Q4) and GER-5FPF+GER-5MRPR (Q5) (Bisht *et al.,* 2009; Pushpa *et al.,* 2015) got amplified in all the parents.

In the present study for faster results and to reduce the use of more resources, two molecular markers GER1 and GER2 were taken for screening the selected F_2 population.These markers have been previously reported for screening low glucosinolate lines in *B.juncea* (Singh *et al.,* 2010; Banga *et al.,* 2009). Further, they have been validated extensively by others (Pushpa *et al.,* 2016) where the maximum contribution (71%) to phenotypic variance was an attribute of theses markers.

The marker GER1 carrying the gene *BjuA. Gsl*-ELONG.a mapped to QTL *J2Gsl*1 (Bisht *et al.,* 2009; Pushpa *et al.,* 2015) gave amplified product of size 650 bp in PDZ-1, RLC-3 and HEERA and 950 bp in NRCHB101, DRMR150-35 (Fig. 3a).

This is intune with previous report by Pushpa *et al.,* (2015) where, low total glucosinolate content lines show amplicon size of 650 bp and for high total glucosinolate content its amplicon size is 950 bp. The marker GER-5 carrying the gene *BjuA. Gsl*-ELONG.c,d mapped to QTL *J3Gsl*2(Bisht *et al.,* 2009; Pushpa *et al.,* 2015)gave amplicon size of310 bp among PDZ-1, RLC-3 and Heera and 350bp in NRCHB101 and DRMR150-35 (Fig. 3b). Our report is in agreement with previous researcher Pushpa *et al.,* (2015) where, low total glucosinolate content lines show amplicon size of 310 bp and high total glucosinolate content showed amplicon size of 350 bp.

Confirmation of these two markers in all the parents leads us to further screening of selected F_2 populations. From the clustering data (Fig. 2a-f) we selected lines having less than 30 µmol/g total glucosinolate content (Fig. 2b and 2d) and validated using GER-1 and GER-2 markers. As expected two bands were observed as they are F_2 progenies. This confirmed our selection to be in the right track and these lines will be further utilized for the next generation. According to Bisht *et al.,* (2009) the *BjuA. Gsl*-ELONG.a and *BjuA. Gsl*-ELONG.c,d are responsible for chain elongation of glucosinolates. This implies chain elongation have a lot to do with the variation in phenotype like glucosinolate content.

In the present experiment, the seed samples from each cross were used to measure glucosinolate content. We could observe markable variation in glucosinolate content and also glucosinolate content in some of the F_2 plants was lower than that of the maternal parents.

This indicates that glucosinolate content could be reduced if high glucosinolate B. *juncea* genotypes are intercrossed with low glucosinolate genotypes. The use of already reported markers has fastened the breeding programme in understanding the segregating lines. Thus the results obtained in this study will further help the breeders in crop improvement and breeding programme for producing high-yielding quality hybrids in *B. juncea*.

Conflict of interest

All the authors do not have any interest of conflict

Acknowledgement

Funding agency Indian Council of Agricultural Research – CRP-Molecular Breeding, Director ICAR-DRMR, for providing necessary facilities for carrying out this work. Dr. A.K. Singh, Genetics Division, Indian Agricultural Research Institute, New Delhi and Dr. Bishal Gurung from ICAR-Indian Agricultural Statistical Research Institute, New Delhi for helping us with statistical analysis are highly acknowledged.

Author's contribution

V. V. Singh designed the experiment, conducted field experiments and prepared the manuscript. Monica Dubey and Ibandalin Mawlong estimated total glucosinolate content and prepared the manuscript. Monica Dubey and Pawan Paliwal carried the molecular analysis. M.S. Sujith Kumar helped in drafting the manuscript.

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How to cite this article:

Singh, V.V., Ibandalin Mawlong, Monika Dubey, Pawan Paliwal and Sujith Kumar, M.S. 2018. Variability in Total Glucosinolate Content in F_2 Generation of *B. juncea* and their Molecular Validation. *Int.J.Curr.Microbiol.App.Sci.* 7(12): 3346-3357.
doi: https://doi.org/10.20546/ijcmas.2018.712.385