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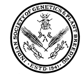

Validation of molecular markers linked to low glucosinolate QTLs for marker assisted selection in Indian mustard (*Brassica juncea* L. Czern & Coss)

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Abstract

Six earlier reported markers closely linked to low glucosinolate QTLs of *Brassica juncea*, spread across 'A' genome (A2, A3 and A9) were validated in a recombinant inbred line (RIL) population of a cross between Pusa Mustard-21 (low erucic acid) and EC-597325 (double low) genotypes, to utilize them in marker-assisted selection (MAS). Of them, four markers *viz.*, GER 1 amplified alleles of 650 bp and of 950 bp, GER 5 amplified 310 bp and 350 bp, At5gAJ67 amplified 500 bp and 450 bp and Myb28 amplified alleles of size 900 bp and 920 bp in EC597325 and Pusa Mustard-21, respectively and therefore differentiated low and high glucosinolate parents. These four polymorphic markers were then used to genotype the phenotyped RIL population consisting 608 plants. Marker-trait association was tested for goodness of fit using $\chi 2$ test. Of the four markers, GER1 and GER5 showed higher phenotypic variance (R^2 value) compared to the others, indicating their significance in determination of glucosinolates and prospects for use in MAS for development of Indian mustard genotypes with low glucosinolates content.

Key words: Indian mustard, oil quality, molecular markers, glucosimolates, QTLs

Introduction

Among the nine annual oilseed crops grown in the country, *Brassica* ranks second in importance contributing about 30% to the total oilseed production (Kumar et al. 2012). Quality in oilseed Brassicas is defined by the improved fatty acid profile of the oil and low total glucosinolates in the seed meal cake. Oil is extensively used for cooking and seed meal cake, a rich source of proteins and minerals with well balanced amino acid and vitamin E, is used as poultry and animal feed. Despite its nutritional benefits, use of seed meal cake as a protein supplement to poultry and animal feed is limited. This is mainly due to presence of high concentration of total glucosinolates that causes nutritional disorders and toxicity in animals and birds (Fenwick et al. 1983, Griffiths et al. 1998). Glucosinolates themselves appear to have very little anti-nutritional effects, however, physical tissue or cell injury leads to the breakdown of glucosinolates through the hydrolytic action of the enzyme myrosinase. Derivative compounds of glucosinolates have a wide range of biological functions including anti-carcinogenic properties in humans and anti-nutritional effects of seed meal in animals (Rosa et al. 1997; Mithen et al. 2000; Mithen, 2001). Thus, the presence of high amount of glucosinolates (80-160 micromoles/g) in seed meal cake restricts the global utilization of Indian mustard (*Brassica juncea*) cake.

Efforts directed towards development of high yielding varieties possessing low erucic and/or low glucosinolates through pyramiding the responsive genes/QTLs separately or in combination led to good number of single and double zero genotypes (Singh et al. 2015). However, due to apparently complex nature of glucosinolate inheritance, breeding elite varieties with low total glucosinolates through backcross programs require tedious biochemical assay and could take several years. Moreover, very low probability of recovering low-glucosinolate progeny in subsequent segregating generations is compounded by the risk of cross pollination with other high/low-glucosinolate

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breeding lines. Development of molecular markers, to monitor seed quality features in progenies, has greatly improved efficiency of plant breeding (Naresh et al. 2012). Molecular markers which could identify low glucosinolate alleles in seedling or vegetative tissues of the segregating generations would greatly enhance the possibilities of introgressing these alleles in desirable genotypes with high glucosinolates content.

Attempts on identification of DNA markers based on linkage analysis revealed location of several glucosinolate loci/QTLs (Ramchiary et al. 2007, Bisht et al. 2009). QTLs being highly sensitive to environment and genetic background, its validation usually refers for the verification that a QTL is effective in different genetic backgrounds (Langridge et al. 2001). Various studies have shown several DNA markers linked with specific glucosinolate genes that may be useful in marker assisted selection (MAS) in segregating progenies (Bisht et al. 2009). To utilize the available information on these markers effectively for development of genotypes with low level of total glucosinolates, these markers need to be validated. The present study, therefore, was undertaken to validate the already reported markers linked to glucosinolate trait using selected recombinant inbred line (RIL) population of *B. juncea*.

Materials and methods

A set of 608 Recombinant Inbred Lines (RILs) in F_8 generation developed by single seed descent method from a cross between a low erucic acid variety Pusa Mustard-21 (glucosinolates >80 μ moles/g defatted seed meal cake) and a double low exotic genotype EC597325 (glucosinolates <30 μ moles/g defatted seed meal cake) was evaluated during *rabi* 2011-12 at ICAR-Indian Agricultural Research Institute, New Delhi. DNA extraction was carried out on the fully expanded, young leaves collected from each genotype at 5-7 leaves stage of the crop which was attained at three weeks after sowing. Genomic DNA was isolated using the standard cetyl trimethyl ammonium bromide (CTAB) protocol as described by Murray and Thompson (1980), later on modified by Doyle and Doyle (1990).

Genotyping

Parental polymorphism was studied using six molecular markers of which four markers *viz*., GER1, GER5, Myb28 and At5gAJ67 (Table 1) developed by Bisht et al. (2009). The polymorphic primers were then used to survey the homozygous RILs. PCR amplification was carried out with 25ng of genomic DNA, 0.2 units of Taq DNA polymerase, 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 sterile distilled water. Amplification was carried out in Eppendorf Thermal cycler following initial denaturation at 94°C for 5 minutes, cyclic denaturation at 94°C for 60 seconds, primer annealing at 54-65°C for 60 seconds and the primer extension at 72°C for 2 minutes. The cycle was repeated 35 times with final extension at 72°C for 7 minutes. The amplified PCR products were resolved in 3.5% metaphor agarose gel and visualised with ethidium bromide staining. The genotypic dataset was generated based on PCR amplification profile by scoring presence and absence of specific allele with specific base pair (bp) size for all samples. Chi square (χ^2) analysis for understanding the inheritance pattern of the markers and analysis of variance for testing the significance of marker-trait association were carried out

Phenotyping

Total glucosinolates content of 608 individual plants of RILs and the parents were estimated by using the method based on complex formation between glucosinolate and Tetra chloro palladate (II) as described by Thies (1982) and modified by Kumar et al. (2004). Oven dried seeds (200 mg) were crushed and transferred to a test tube (2 ml) and 300 μl of 70 per cent ethanol was added to inactivate myrosinase and decompose the tissues. In each set, a blank without sample was also included. The tube was incubated in water bath at 80°C for 5 minutes. Then 1.5 ml of double distilled water was added to each sample and was again incubated in water bath for 15 minutes at 80°C. The samples were cooled at room temperature for 15 minutes followed by centrifugation at 5000 rpm for 15 minutes at room temperature. For sample analysis, 5 μl of supernatant was transferred to microtiter flat bottom plate along with blank. 300 μl of sodium tetra chloro palladate [$Na_2(PdCl_4)$], a colour reagent was added to each samples and kept in the oven at 70°C for 30 minutes for the formation of the colour complex. The plate was analysed at 405 nm by SoftMax® Pro 5.4 ELISA Reader.

Results and discussion

Present study, aimed at validation of earlier reported markers for total glucosinolates in Indian mustard. Parents *viz*., Pusa Mustard 21 (high glucosinolates) and EC 597325 (low glucosinolates) varying for total glucosinolates content, and derived biparental RIL

population consisting of 608 lines were investigated in this study. All the primers reported by Bisht et al. (2009) got amplified in the parents, whereas, only four markers linked to A genome QTLs for glucosinolate genes *viz.*, *J2Gsl*1 harbouring *BjuA.GSL-ELONG.a, J3Gsl2* harbouring *BjuA.GSL-ELONG.c,d, BjuA.Myb28.a* and *J9Gsl3* in the vicinity of *BjuA.GSL-ALK.b* genes were found polymorphic between the parents. The marker GER1 (*J2Gsl1*; A2) amplified alleles of size of 650 bp in EC597325 and 950 bp in Pusa Mustard 21 (Fig. 1a) whereas alleles amplified by marker GER 5 (*J3Gsl2*; A3) were 310 bp in EC597325 and 350 bp in Pusa Mustard 21 (Fig. 1b). At5gAJ67 (*J9Gsl3*; A9), the third marker amplified allele of 500 bp in EC597325 while in Pusa Mustard 21, an allele of 450 bp was amplified (Fig. 1c). The marker Myb28 (*J3Gsl2*; A3) amplified 900 bp fragment in EC597325 while the size of allele amplified by the same marker was 920 bp in Pusa Mustard 21 (Fig. 1d). These four markers were then used for genotyping 608 RILs derived from above two genotypes. RILs with these four loci, when present in recessive form, had total glucosinolates content below 30 µ moles per gram of seed. This justifies the practical utility of the marker system for development of genotypes with low glucosinolates. For effective use of these markers in selecting low glucosinolate genotypes without false positives, marker-trait association was tested for goodness of fit by χ^2 test. Non-significance of χ^2 values indicated that all the polymorphic markers were following Mendelian inheritance pattern (Table 2).

Phenotypic variance (R^2) was calculated for all the polymorphic markers linked to the glucosinolate QTLs to understand the extent of trait contribution by each locus. The markers GER1 linked to *J2Gsl1* and GER5 linked to *J3Gsl2* showed high R^2 of 0.46 and 0.25 respectively, compared to other two markers

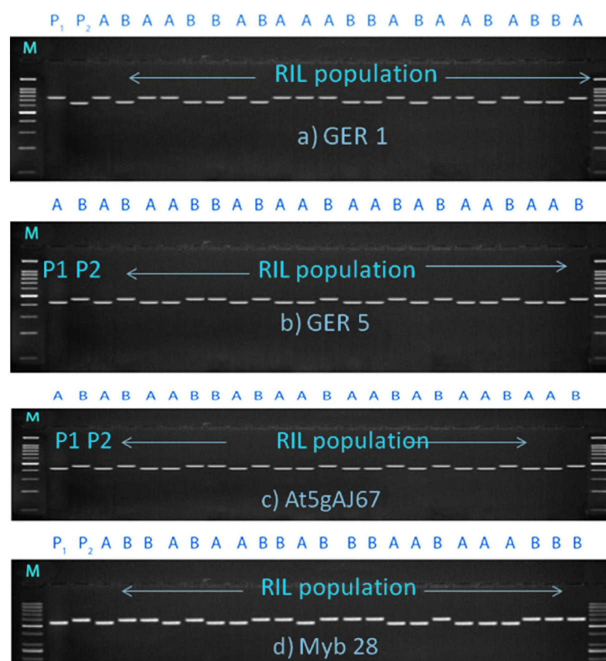

Fig. 1. Genotyping of RILs using polymorphic markers: (a) GER1, (b) GER5, (c) At5gAJ67 and (d) Myb 28; M-100 bp ladder; P_1- Pusa Mustard 21; P_2- EC597325; A- RILs with P1 type allele; B- RILs with P2 type allele

At5gAJ67 (R^2: 0.03) and Myb28 (R^2: 0.03) (Table 3). This clearly indicates that these two markers namely GER1 and GER5 are together responsible for 71 per cent variation in glucosinolate content. Pyramiding of four genes using marker-assisted backcross breeding necessitates identification of plants -heterozygous for four loci in backcross generation (BC_nF_1) and -homozygous for four loci in selfed generations (BC_nF_2). This requires larger population to be genotyped to identify the desirable plant with favourable genetic constitution at all four loci. Since the study showed

Table 1. Details of known glucosinolate QTLs linked markers used for validation in RIL population derived from the cross Pusa Mustard 21 x EC597325

Linkage group	QTL	Primer code	Co-localised candidate gene (s)	Reference
A2	*J2Gsl1*	IP3 GER1 GER1-MRP	*BjuA.Gsl-ELONG.a*	Bisht et al. 2009
A3	*J3Gsl2*	Myb28-F Myb28-R	*BjuA.Myb28.a.*	Bisht et al. 2009
		GER5-FP GER5-MRP	*BjuA.Gsl-ELONG.c,d*	Bisht et al. 2009
A9	*J9Gsl3*	At5gAJ67-F At5gAJ67-R	—	Bisht et al. 2009

Table 2. Segregation of co-dominant SSR markers in the RIL population of Indian mustard

Marker	Genotypes with marker allele			
	A	B	χ^2 (1:1)	
GER1	308	300	0.105263	χ^2 1, 1%
GER5	298	310	0.279605	= 6.635
Myb28	318	290	1.289474	
At5gAJ67	322	286	2.131579	

that two of the markers had high coefficient of determination, introgression of these two will be much more easy and also effective. With less population size, time and resource the desirable segregants can be easily identified. Moreover, two of the markers with high phenotypic variance co-localise with the genes *BjuA.Gsl-ELONG.a* (GER1) and *BjuA.Gsl-ELONG.c,d* (GER5) (Table 1). This shows that the *Gsl-ELONG* gene family could play a major role in altering the synthesis of glucosinolate in the seed by controlling the C4 and C5 chain elongation pathway. The same was also reported by Bisht et al. (2009), that genes controlling chain elongation (*BjuA.Gsl-ELONG.a* and *BjuA.Gsl-ELONG.c,d*) play vital role in effecting low glucosinolate than the *Myb* genes controlling basal level glucosinolate production. Further characterisation of these gene families may lead to identification and fine mapping of candidate genes responsible for effecting low glucosinolate in Indian mustard.

Various DNA marker systems have been used for mapping and tagging of different traits in the *B. juncea*. Earlier it was assumed that use of markers that are linked with the QTLs from preliminary mapping studies can be directly used in MAS. Recent studies on extent of success of introgression of QTLs revealed that validation and/or fine (or high resolution) mapping may be required, as they are highly environment sensitive and expression varies with different genetic backgrounds. QTL validation usually refers to the verification of the effect of a QTL in different genetic backgrounds (Langridge et al. 2001). Many QTLs for individual/total glucosinolates are frequently reported in scientific journals, but reconfirmation of identified QTLs in other genetic background and identification of more useful markers are usually not considered novel enough to warrant new publications. Prior to their use in the marker assisted breeding, the first important thing is the validation of these markers in the potential parental lines and breeding populations. Once the markers for a trait (s) are validated, they can be used effectively in practical plant breeding programme through MAS. Markers used in current study were reported earlier by Bisht et al. (2009) that are PCR based and can be implemented routinely to detect the presence of low glucosinolate QTLs in desirable genotypes. Various marker systems reported for quality traits in different *Brassica* spp. have been validated *viz*., SSR marker (Ol12FO2) linked to methyl sulphinylalkyl glucosinolates was validated in kale

Table 3. ANOVA for single marker analysis for marker-trait association in the RIL population of Indian mustard

Source of variation	df	Sum of Squares	Mean Square	F	Sig.	R	R^2
a. GER 1							
Regression	1	87968.297	87968.3	249.3085	.000	0.676	0.456976
Residual	606	213826.563	52.8491				
Total	607	301794.86					
b. GER 5							
Regression	1	72905.323	72905.32	106.0088	.000	0.498	0.248004
Residual	606	416763.589	687.7287				
Total	607	489668.912					
c. At5gAJ67							
Regression	1	25819.083	25819.08	18.92325	.000	0.16	0.0256
Residual	606	826832.803	1364.411				
Total	607	852651.886					
d. Myb 28							
Regression	1	52630.894	52630.894	52.27318	.000	0.309	0.095481
Residual	606	610146.992	1006.843				
Total	607	662777.886					

(Sarikamis et al. 2010); two major QTLs (*TSWA7a* and *TSWA7b*) identified for seed yield traits in *B.napus* (Shi et al. 2009; Fan et al. 2010). As discussed earlier, expression of QTLs vary with different genetic backgrounds so there is a need to validate marker-QTLs/trait association before exploiting QTLs in MAS. MAS approach is useful for improvement of trait like low glucosinolate, a recessive trait that is expressed to varying levels at the adult-plant stage. With the validation of these four markers (GER1, GER5, Myb28 and At5gAJ67) linked to low glucosinolate trait, MAS can be used as an efficient selection tool by mustard breeders involved in quality breeding programme.

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