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A candidate gene-based association study of introgressed pod shatter resistance in *Brassica napus*

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Yield losses at maturity due to unsynchronized pod shattering remain a major rapeseed breeding challenge. Variation for shatter resistance in the germplasm collections is inadequate for breeding manipulations. We have recently transferred resistance to pod shattering from *Brassica carinata* to *Brassica napus*. Introgression lines (ILs) were phenotyped for shatter resistance using the pendulum machine. Introgressive breeding was successful in enhancing rupture energy in the ILs, which varied from 1.8 to 7.2 milli Joules (mJ) for Environment 1 (E1) and 2.7 to 6.5 mJ for E2 while the corresponding values for natural *B. napus* ranged from 2.2-3.5 mJ (E1) and 2.2-4.3 mJ (E2), respectively. *B. carinata* had average rupture energy of 6.3 mJ (pooled over environments). On the basis of data averaged over two environments, I2 (6.3 mJ), I3 (5.2 mJ), I8 (5.6 mJ), I22 (5.1 mJ), I32 (5.2 mJ) and I41 (5.2 mJ) appeared very promising as germplasm resources for future breeding. Significant marker trait association between candidate gene NAC NAM (no apical meristem, *Petunia*), ATAF1/2 (*Arabidopsis thaliana* activating factor) and CUC2 (cup-shaped cotyledon, *Arabidopsis*) and rupture energy explained 19% of variation for the trait. IND3 (indehescent 3) also appeared to be associated with rupture energy under E1. These polymorphisms serve as encouraging candidates for developing molecular markers useful in marker-assisted deployment of introgressed shatter resistance.

Keywords: Association mapping, *Brassica napus*, Candidate gene, Introgression, Pod shatter resistance

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Brassica napus (AACC; 2n = 4x=38) is an allotetraploid that arose from voluntary hybridizations between *Brassica rapa* (AA; 2n=2x=20) and *Brassica oleracea* (CC; 2n=2x=18), almost 7500 years ago[1]. The parental diploid species themselves evolved from a common progenitor about 3.7 million years ago[2]. *B. napus* (also known as rapeseed or canola) is now a premier oilseed crop of China, Europe, Canada and Australia. It has areas of adaptation in cool environs of north-west India. It contributes almost 15% to the worlds' supply of vegetable oils[3]. Demand for this crop is expected to grow further in view of the burgeoning requirements of vegetable oils for food and fuel. Due to its significant economic importance, the crop is the focus of intensive international efforts to increase productivity and improve quality. Notwithstanding impressive gains in productivity and seed quality, yield losses at maturity due to unsynchronized pod shattering remain a crop breeding challenge. Controlled pod shattering is critical for limiting yield losses.

There is little variation for this trait in the germplasm collections[4] and the level of protection available is inadequate to circumvent windrowing[5]. Peng *et al.*[6] used ripping method to study shatter resistance in 220 rapeseed lines. The ripping force varied between 0.59N to 2.75N. Digenic inheritance and moderate heritability (50%) were also suggested. In *B. rapa*, shatter resistance appeared to be conditioned by 2-3 genes[7]. Loci for shatter resistance have also been mapped[8]. Raman *et al.*[9] used a genotyping-by-sequencing approach (DArTSeq) to discern the genetic divergence for resistance to pod shatter in a large *B. Napus* collection. They determined 12 significant QTLs on chromosomes A-3, -7, -9 and C-3, -4, -6, -8 that collectively elucidated for ~ 57% of the variations for pod shatter resistance. They further opined that divergence for shatter resistance genes in *B. napus* was narrow; due possibly to the absence of favorable allele combinations for pod

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shatter resistance in the parental species that participated in initial hybridization events. It is also likely that alleles associated with indehiscent pods were not selected for during its domestication process.

Pod structure in *Brassica* involves two valves enfolding the seeds. Valves were joined together with valve margin cells through the replum. These separate at maturity allowing the valves to detach from the replum and release the seeds. This is facilitated by a separation layer in the pod suture[9]. The valve margins form narrow stripes of a lignification layer (LL) and a separation layer (SL). This specialized structure facilitates fruit opening and the efficient release of seeds. In spite of the long history of divergence[10], pod structure and development in *B. napus* remains essentially the same as that in *Arabidopsis.* Specific genes controlling resistance to shatter have been identified in *Arabidopsis*. Most of the mutants or natural variants with indehiscent fruit in *Arabidopsis* result frequently from the loss-of-function or loss-of-expression of genes involved in the regulation of cell identity of lignified valve margin and abscission layers[11].

Shatterproof1 (*SHP1), Shatterproof2 (SHP2*)[12], *NAC* (*NST1* and *NST3*)[13], the basic-helix-loop-helix protein genes *Indehiscent* (*IND*)[14] and *Alcatraz* (*ALC*)[15] are the genes encoding transcription factors that are fundamental for differentiation of LL and SL. The genes expressed in valves (*REPLUMLESS* (*RPL*) and *FRUITFULL* (*FUL*) inhibit the expression of valve-margin identity genes[16]. *IND* is also negatively regulated by *FRUITFULL (FUL)* and it prevents valve margin cells from adopting a valve identity. Over expression of a MADS BOX gene from *B. juncea* and *FUL* gene from *B. napus* have been reported to enhance pod shatter resistance in *B. napus*[17]. *SHAT1*, *Shattering1* (*Sh1*), *SH4* and *RPL* genes have been shown to confer natural variation for resistance to shatter in rice, sorghum and wheat[18]. Loss of fruit dehiscence as a derived, morphologically adaptive character has been reported from many lineages[19].

Due to a very limited variation in *B. napus*, sources of resistance to pod shatter have been sought from *B. juncea* and *B. carinata*, with little or no success[20]. Absence of allosyndetic pairing between the B-genome chromosomes from *B. carinata* and A-/C-genome chromosomes from *B. napu*[21] may have been the limiting factor for failure of these efforts. We have transferred resistance to pod shattering from *B. carinata* to *B. napus*[22]. We succeeded it with backcrossing, selfing and phenotypic selection. The number of backcrosses was limited to two which assisted in retaining higher proportion of donor genome (B-genome). Five cycles of synchronous selfing followed. In each selfing cycle, retention of B-genome genetic information was assured by using molecular markers and phenotypic selection for hard to thresh siliquae. For achieving this, a very large population base in each selfing cycle was imperative. Each one of the introgression lines is a *euploid* (2n=38) and carries B-genome introgressions as confirmed by fl-GISH and molecular markers[23].

In the present communication, we report genetic variation for resistance to shatter in newly developed *B. napus* introgression lines compared with 15 natural *B. napus* accessions. Shatter resistance was measured using the pendulum machine. We also report our inferences from association mapping using candidate gene-based approach to genic markers that are associated with shatter resistance. We expect that mapping quantitative trait loci (QTLs) linked to pod shatter resistance will help to reduce the linkage drag that may be associated with introgressed variation and assist in developing rapeseed cultivars, through a molecular marker-assisted selection (MAS) strategy, which are suitable for mechanical harvesting. To our knowledge, this is the first report for such study in rapeseed so far.

Material and Methods

Plant material

A panel of 96 genotypes, which included 81 BC_1S_6 introgression lines, 14 recipient *B. napus* genotypes and a standard check cultivar, was raised during the 2012-13 cropping season at two dates of sowings {October 15, 2012 (timely-sown Environment 1) and November 15, 2012 (late-sown Environment 2)} in an alpha lattice design with two replicates. The two dates of sowing were thus treated as two different environments. The crop was raised as per the recommended agronomic practices.

Evaluation for resistance to shatter

B. napus introgression lines with euploid chromosome number (2n=38) were selected to assess pod shatter resistance. Donor parent for the trait, *B. carinata*, and normal *B. napus* recipient genotypes were used as standard checks. Five plants were randomly tagged per plot per replication per environment. Five pods from the middle of the main raceme from each tagged plant were then carefully

detached after these had attained physiological maturity. The pods were kept in 15 mL conical bottom Tarson tubes that contained approximately 1 g coarse silica gel blue self-indicating granules to bring the pods to constant moisture content for storage at room temperature. The pods were dried at 70°C for 24 h in a hot air oven before assessing their shattering strength. The corresponding resistance to pod shatter of each genotype was measured in terms of rupture energy (RE) using an improvised pendulum apparatus fabricated in Australia. It is based upon the amount of energy lost principle after the pendulum strikes the pod with a known force to split it open[20]. The lost energy is recorded as that required for rupturing a pod.

Candidate gene primers

The introgression lines, along with the donor and recipient parents, were probed with the primers developed from the sequence information of the genes putatively associated with pod shattering. The candidate genes were SHP1 (Shatterproof1), SHP2 (Shatterproof2), NST1 (NAC secondary wall thickening promoting factor 1), IND (Indehiscent) and PG (Polygalacturonase). The candidate gene-specific primers SHP1 and SHP2 were identified using SSR PRIMER 3 software. We used previously reported primer sequences for other candidate genes[9].

Genotyping studies

DNA was extracted from young leaves taken from a single plant of each genotype by CTAB extraction method[24] with marginal modifications[25,26] and was suspended in TE buffer (pH 8). It was digested with RNaseA at 37°C for 1 hr and its quantity was assessed by spectrophotometric analysis using a biophotometer (Eppendorf Bio Photometer Plus, Eppendorf, Germany). The DNA was then diluted to a concentration of 5 ng/µL before conducting PCR assay. Template DNA (5 µL of 5ng/µL) was added to 15 µL of master mix that contained 1.0 µL 10x reaction buffer, 2.0 µL 2.0 mM dNTPs, 1.0 µL 1 mM forward primer, 1.0 µL 1 mM reverse primer, 0.3 µL Taq polymerase and 4.7 µL Millipore sterilized water. The standard SSR protocol (1 cycle of 4 min at 94°C; 35 cycles of 1 min at 94°C, 30 s at T_A, 30 s at 72°C; 1 cycle of 7 min at 72°C and a final hold at 4°C) was followed for PCR analyses of these primers. *In-vitro* amplification using PCR was performed in 384 welled plate in Applied Biosystems (Model EN61328) PCR and 96 welled plate in Eppendorf AG (Model 6325) PCR. Automated high throughput electrophoresis system (Caliper Lab Chip GX version 3.0.618.0) and 3.5% agarose gel electrophoresis were used to separate the PCR products, which were automatically sized.

Statistical analysis

For each accession, mean values of rupture energy were calculated for both the environments. SAS version 9.2[27] was used to perform Analysis of variance (ANOVA) using PROC MIXED for examining the genotype, environment and genotype × environment interactions. STRUCTURE version 2.2.3[28] was employed to deduce the population structure of the test genotypes using the admixture model and correlated allele frequencies. The burn-in length period between 1 and 10 subpopulations (*K*) was of 100,000 iterations, followed by 100,000 Markov Chain iterations. Principal component analysis (PCA) was determined by the polymorphism data generated by CG-SSRs. For association analysis, first and second principal components were used (*D* matrix). DNA polymorphisms were analyzed for association with rupture energy, separately for both the environments. Polymorphisms with a minor allele frequency of < 5% were excluded from association studies. To analyze associations between polymorphic sites and rupture energy, GLM (general linear model) and MLM (mixed linear model) were used. The analysis was conducted with the software TASSEL[29]. Q matrix[38] and kinship matrix[30,31,32] were used for removing effects of the structure on the association panel and relatedness among the genotypes. Adjusted *p*-value (Bonferroni correction) of less than 0.05 was accepted for declaring an association significant. To estimate phenotypic variation explained by a particular marker, R^2 values were calculated[33]. The increments estimated proportions of explained variance for individual markers in R^2 statistic after fitting fixed individual markers in a model with fixed covariate for population structure.

Results

Assessment for pod shatter resistance

The variation for pod shatter resistance is depicted in the form of box plots (Fig. 1) and presented in Table 1. The rupture energy for the introgression lines varied from 1.8 to 7.2 mJ under timely-sown environment E1 and from 2.7 to 6.5 mJ under late-sown environment E2. *B. carinata,* the resistant parent, had average pod rupture energy of 6.4 mJ (E1) and 6.2 mJ (E2), respectively. The average pod rupture energy in the introgression lines under the first environment ranged between 1.8 mJ (I-36) and

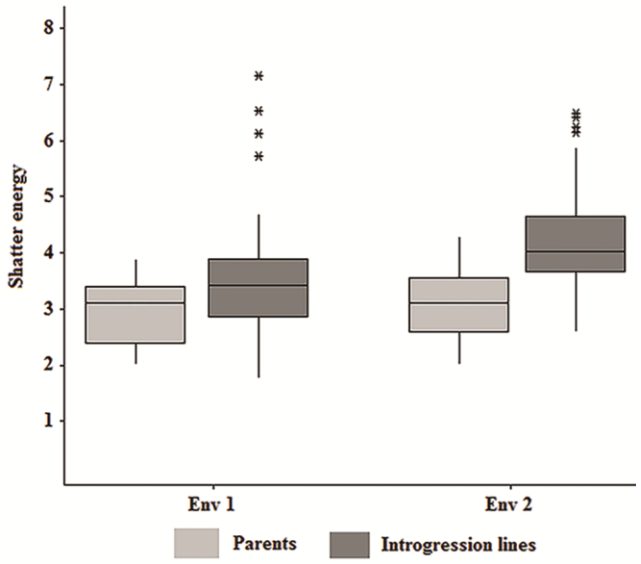

Fig. 1 — Box plots depicting the quantum of phenotypic variation for resistance to shatter in *B. napus* parents and introgression lines

7.2 mJ (I-41) while under the second environment the range was between 2.7 mJ (I-63) and 6.5 mJ (I-2). Introgression lines with very high rupture energy under environment E1 were I-2 (6.1 mJ), I-32 (5.7 mJ), I-41 (7.2 mJ) and I-68 (6.5 mJ). Under environment E2, introgression lines with high rupture energy were I-2 (6.5 mJ), I-3 (5.9 mJ), I-8 (6.4 mJ), I-12 (5.1 mJ), I-16 (5.4 mJ), I-20 (5.4 mJ), I-22 (5.5 mJ), I-23 (5.4 mJ), I-35 (5.2 mJ), I-40 (5.9 mJ), I-52 (6.2 mJ), I-56 (6.3 mJ), I-59 (5.7 mJ), I-67 (5.7 mJ), I-71 (5.3 mJ), I-72 (5.2 mJ) and I-74 (5.8 mJ). Based onthe data averaged over two environments, I-2 (6.3 mJ), I-3 (5.2 mJ), I-8 (5.6 mJ), I-22 (5.1 mJ), I-32 (5.2 mJ) and I-41 (5.2 mJ) appeared very promising. Many introgression lines either equaled, or were close to, the *B. carinata* check for their shatter resistance. A weak negative correlation existed between pod rupture energy and pod length (-0.27, $p \leq 0.009$) and seeds per pod (-0.05, $p \leq 0.61$), respectively (data not

Table 1 — Average pod shatter energy (mJ) measured in the 96 test genotypes (Introgression Lines (ILs) and *B. napus* parents) under two environments (dates of sowing).

Genotype	Pod shatter energy		Genotype	Pod shatter energy		Genotype	Pod shatter energy	
	E1	E2		E1	E2		E1	E2
I-1	4.3	4.2	I-33	3.1	3.8	I-65	3.0	3.4
I-2	6.1	6.5	I-34	3.2	3.7	I-66	2.9	3.4
I-3	4.6	5.9	I-35	4.4	5.2	I-67	3.8	5.7
I-4	3.8	3.8	I-36	1.8	3.2	I-68	6.5	3.9
I-5	2.8	4.0	I-37	2.8	4.2	I-69	2.8	3.4
I-6	2.8	2.9	I-38	2.0	3.4	I-70	2.2	4.6
I-7	3.9	4.3	I-39	3.7	4.4	I-71	3.2	5.3
I-8	4.7	6.4	I-40	3.2	5.9	I-72	3.8	5.2
I-9	4.6	4.4	I-41	7.2	3.4	I-73	3.7	3.8
I-10	2.8	4.5	I-42	3.6	4.5	I-74	3.1	5.8
I-11	2.0	2.6	I-43	2.5	3.6	I-75	3.5	3.1
I-12	4.2	5.1	I-44	4.2	4.3	I-76	3.1	4.9
I-13	2.4	4.4	I-45	3.6	4.0	I-77	3.4	3.9
I-14	3.4	3.8	I-46	3.4	4.3	I-78	2.8	3.4
I-15	3.7	4.0	I-47	4.6	3.7	I-79	4.4	4.0
I-16	3.7	5.4	I-48	4.2	4.0	I-80	3.6	4.0
I-17	3.1	3.8	I-49	3.5	4.2	I-81	3.1	3.9
I-18	3.2	4.7	I-50	3.6	3.7	CHARLTON	2.3	2.3
I-19	3.1	4.2	I-51	2.6	3.9	GSC6	3.1	3.4
I-20	3.4	5.4	I-52	3.4	6.2	MONTY	2.4	2.6
I-21	3.7	4.0	I-53	4.0	3.8	MYSTIC	3.2	3.3
I-22	4.6	5.5	I-54	2.7	4.2	RAINBOW	3.3	4.2
I-23	3.6	5.4	I-55	3.6	3.4	RR-001	2.5	2.7
I-24	2.1	3.9	I-56	4.2	6.3	RR-002	3.7	3.6
I-25	3.3	3.2	I-57	4.3	4.5	RR-005	3.9	4.3
I-26	2.4	4.2	I-58	2.8	3.3	RR-009	3.4	3.1
I-27	2.4	3.7	I-59	2.4	5.7	RR-013	3.4	3.1
I-28	2.9	4.6	I-60	3.7	3.6	RQ-001	3.1	3.0
I-29	3.6	2.9	I-61	3.4	3.6	RQ-011	3.0	3.2
I-30	2.9	3.5	I-62	3.7	3.8	SKIPTON	2.2	2.2
I-31	3.1	4.3	I-63	4.4	2.7	SURPASS 400	3.6	4.3
I-32	5.7	4.8	I-64	3.2	4.4	TRIGOLD	2.0	2.0

included). To study the Genotype × Environment interaction, a GGE biplot analysis was conducted (Fig. 2). The first date of sowing showed long vector and the second date of sowing had a short vector. Hence first date of sowing can be considered better to document variation for rupture energy. There were a large number of introgression lines that fell closer to the origin in the centre of polygonal. These genotypes show average trait performance and may not contribute to G × E interaction. The distance between two genotypes on the scatter plot is known to approximate the Euclidean distance between them and, therefore, is a measure of dissimilarity. Introgression lines I-54, I-75, I-80 and I-81 appeared most distinct (Fig. 2).

Polymorphism assays

For all the five candidate genes that amplified in the association panel, PCR products of the expected fragment size, with minor deviations, were detected. Polymorphism was detected for SSRs associated with various regions of the five candidate genes. The distinguished polymorphisms were characterized by their minor allele frequency, that is, the recurrence frequency at which the less regular allele of a polymorphism happened in the association panel. A 5% threshold was used for the five candidate genes. The genotyping with cg-SSRs permitted a scoring of 21 alleles.

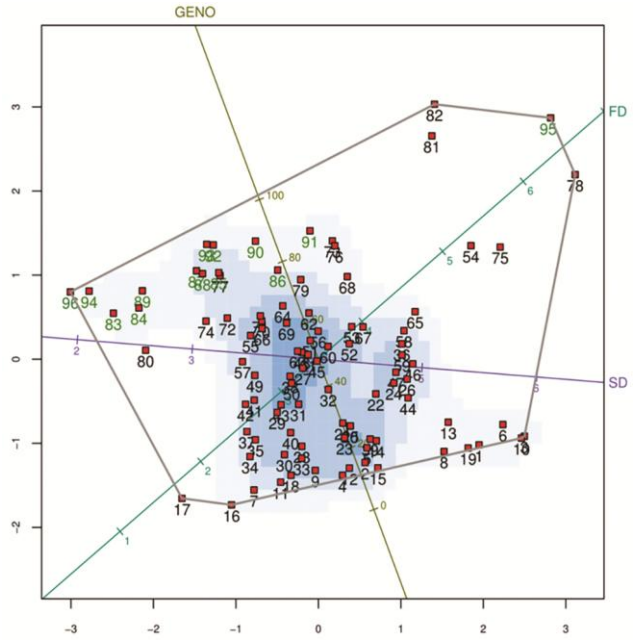

Fig. 2 — GGE Biplot representing the Genotype x Environment interaction for resistance to pod shatter

Population structure and differentiation

For the 96 genotypes, population structure was construed using a model-based software STRUCTURE by setting the number of clusters (K) from 1 to 10 with five replications for each K. The highest ΔK value was observed at K=3, there by assigning the 96 genotypes into three major groups. The tree-based analysis and STRUCTURE analysis results were very similar. The pair wise kinship estimates based on molecular markers conceded that ~53% genotype pairs had high kinship values; this implies the involvement of some common parents in the breeding history of these germplasm groups. This might be credited to the introgressive breeding that generated a broad range of genetic variation.

Linkage disequilibrium

Pairs of segregating sites were investigated to better assess LD in candidate genes and the probability of correlation of CG-SSRs with other adjacent genes (Fig. 3). One strong LD block was observed for the candidate gene NAC loci, one each in NAC 5, 6, 7, 8 and 9. Low LDs were also observed for NAC3 and IND3. Data from all the five candidate gene loci were pooled to estimate the overall decay of LD which is shown by plots of r^2 as a function of physical distance in base pairs between the SSRs

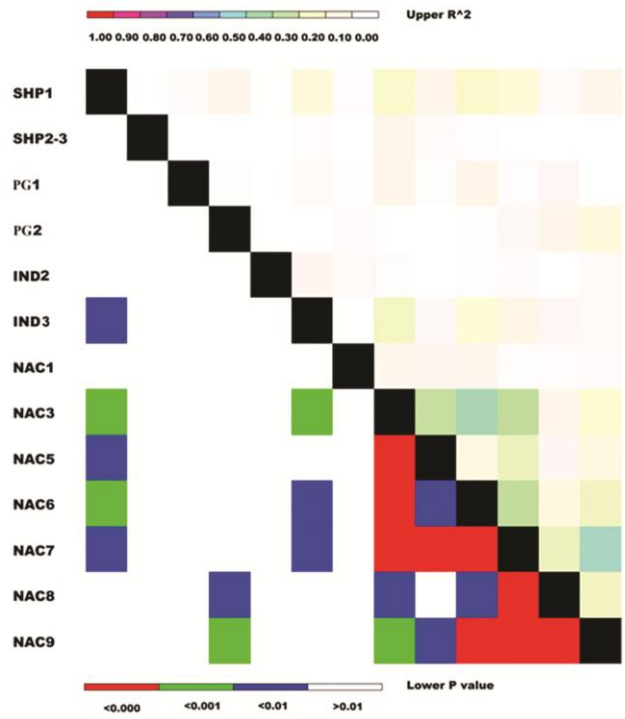

Fig. 3 — Linkage disequilibrium in the genomic regions associated with candidate genes.

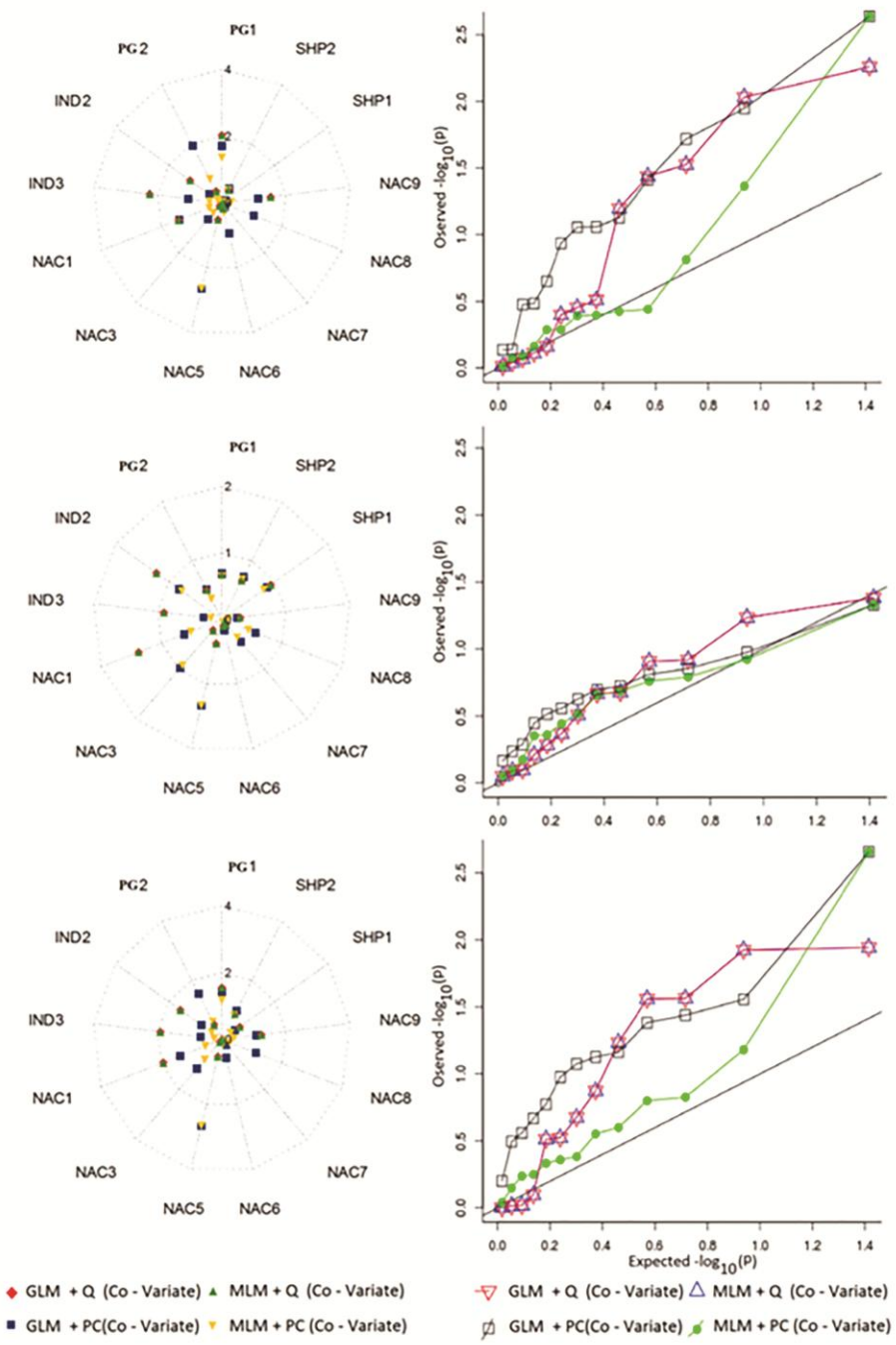

Fig. 4 — Association profiles showing significant markers associated with rupture energy parents and introgression lines

(Fig. 3). The r^2 was 0.0132 and only 1.83% of the total possible marker locus pairs were in significant LD ($p<0.001$).

Marker loci associated with rupture energy

All polymorphic sites were included for association analysis of the candidate genes and rupture energy. Several association models were used to institute marker-trait associations and to account for both Type 1 and Type 2 errors. These included GLM, MLM, GLM+Q matrix, MLM+Q matrix, GLM+PC and MLM+PC (Fig. 4 and Table 2). QQ plots revealed better fits for GLM+PC and MLM+PC (Fig. 4). These models were then used to draw inferences for marker-trait associations. For the timely-sown environment

Table 2 — Association of candidate gene markers with pod rupture energy in introgression lines of *B. napus*

Environment	Method	Co-variance	Locus	P_Marker	Likelihood	df_Model	MS_Error	R^2
Pooled	GLM	PC	NAC5	0.0022*		6	0.5773	0.1915
E1	GLM	PC	NAC5	0.0023*		6	0.8289	0.1801
E1	GLM	Q	IND3	0.0055		4	0.8872	0.1020
E1	GLM	Q	PG1	0.0093		4	0.8967	0.0924
Pooled	MLM	PC	NAC5	0.0022*	-9.84E+01			
E1	MLM	PC	NAC5	0.0023*	-1.14E+02			
E1	MLM	Q	IND3	0.0055	-3.76E+02			
E1	MLM	Q	PG1	0.0093	-2.87E+02			

*Significant at 0.05 (Bonferroni correction) with threshold as 0.003846.

E1, both these models revealed significant marker-trait associations between NAC1 & NAC 5 and rupture energy at a Bonferroni corrected threshold of 0.003846. For the late-sown environment E2, the marker-trait association could not be established for any locus. Association of NAC 5 with rupture energy observed under E1 was confirmed when the rupture energy data for both the environments were pooled. This marker could explain almost 19% of the variation for the trait. IND 3 also appeared to be associated with rupture energy under E1 but the threshold value was marginally low (Fig. 4).

Discussion

Pod burst after fruit ripening is an excellent mechanism of seed dispersal for maximizing survival and adaptive potential of the wild species. In domesticated crops, the unsynchronous fruit dehiscence is unacceptable due to associated yield losses during harvesting. Therefore, shatter resistance was a key domestication trait selected for in most of the cultivated crops. Resistance to pod shatter somehow seems to have escaped the attention of rapeseed domesticators. Improving pod shatter resistance, therefore, is an essential objective of rapeseed breeding but the absence of variation for the trait in the current germplasm has so far prevented the development of shatterproof cultivars. Apart from lack of heritable variation, a significant limitation has been the imprecise evaluation of shatter resistance which was mainly based on field observations such as visual scoring of percent seed loss in terms of seed count after harvest[34] and percent shattered pods[35]. Such assessments tended to be subjective and were not comparable across the institutions and the environments[36]. Availability of a pendulum-based method to assess pod strength by measuring pod rupture energy[20] is a significant development towards a realistic assessment of genetic variability for the trait[37]. Results from this procedure are correlated strongly with estimates of field shattering (r=0.86)[38].

Here in *B. napus*, we introgressed this trait from related non-shattering species *B. carinata*. The present communication details the morphological and molecular assessment of introgressed variation for shatter resistance from *B. carinata* into *B. napus* genotypes. The introgressed variation was characterized through candidate gene-based association studies with the phenotype by scanning the population with shatter resistance-related CG-SSR markers to enhance its practical utility for rapeseed improvement.

Phenotyping introgression lines for shatter resistance clearly showed that introgressive breeding was successful in enhancing rupture energy required for pod shattering. Rupture energy values varied from 1.8 to 7.2 mJ for environment E1 and from 2.7 to 6.5 mJ for environment E2. These values are very significant when viewed in light of corresponding ranges of 2.2 to 3.5 mJ (E1) and 2.2 to 4.3 mJ (E2) for natural *B. napus* genotypes. The introgression lines I-2 (6.3 mJ), I-3 (5.2 mJ), I-8 (5.6 mJ), I-22 (5.1 mJ), I-32 (5.2 mJ) and I-41 (5.2 mJ) appeared very promising as these had rupture energy close or equal to that of *B. carinata.* These can thus be utilized as germplasm resources for future breeding.

Polygon view of a biplot is an ideal way to visualize the interaction patterns between genotypes and environments as it appropriately interprets a biplot[39]. Biplot analysis depicted timely-sown environment E1 as ideal to assess variation for shatter resistance. There were a large number of introgression lines that fell closer to origin in the centre of polygonal. These genotypes showed average rupture energy and may not contribute to GxE interaction. Several genotypes were located near the vertices, which may be considered more responsive to environment interaction as these were placed at the longest distance from the origin.

Although genome-wide association studies are preferred sincethese entail scanning of the whole genome by a large number of markers[40,41], the candidate gene-based studies target genes with known functions in the trait of interest, thereby getting a higher frequency of meaningful trait associations[42]. For our gene-based association studies, we included several *B. napus* shattering orthologs based onhigh sequence homologies to corresponding *Arabidopsis thaliana* genes. *B. napus*is an allotetraploid and therefore, several homologous sequences for each shatter resistance gene of *A. thaliana* were expected. In all, five candidate genes were analysed for polymorphism screening. We used 14 primer pairs to amplify parts of the target genes. Twenty-one fragments were amplified for five candidate genes. Rare polymorphisms (frequency < 5%) were also detected. Future studies would focus onother variations present beyond the amplified regions. An expected outcome was a considerably low polymorphism for SHP1 and SHP2 as compared to other genes since allelic variation for orthologous genes primarily depends on the function of the gene and the germplasm that is utilised for analysis.

B. napus is known to possess an extensive population structure that can confound genetic association studies[43]. STRUCTURE analysis suggested three subpopulations, indicating enough markers for subpopulation calculation. Analysis through PCA confirmed these findings. There was very significant population differentiation between natural *B. napus* and the introgression lines with a region of admixture. Despite a clear population structure, most individuals shared over 50% of their alleles. QQ-plot indicates that the markers adequately modeled population structure and kinship. Given these results, we ran association tests using mixed model and found that marker-trait association between the candidate gene NAC and pod rupture energy under environment E1 was significant and under environment E2 was non-significant. The pooled analysis, however, revealed a highly significant association between NAC and rupture energy. This marker could explain almost 19 percent of variation for the trait. IND3 also appeared to be associated with rupture energy under environment E1, but the threshold value was marginally low. Pod shatter resistance associated with domestication in soybean was recently found to be mediated by NAC gene[44]. The role of NAC gene (NAM, ATAF1/2 and CUC2) has been elucidated in the functional activation of secondary wall biosynthesis and promotion of thickening of FCC secondary walls by expression at 15-fold the level of the wild allele, which is attributed to functional disruption of the upstream repressor. Ectopic expression analyses of IND, PG (*Polygalacturonase*)[45] and FUL *(Fruitfull)*[17] genes have provedthat these genes do govern resistance to pod shatter in *B. oleracea, B. napus* and *B. juncea.*

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Conflict of interest

Authors declare no conflict of interest.

Author Contributions

ID collected the data & wrote the paper; NK performed the analysis; SSB, SB conceived and designed the problem; PS, SSB edited the paper.

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