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ORIGINAL ARTICLE

Anchoring alien chromosome segment substitutions bearing gene(s) for resistance to mustard aphid in *Brassica juncea-B. fruticulosa* introgression lines and their possible disruption through gamma irradiation

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Abstract

Key message **Heavy doses of gamma irradiation can reduce linkage drag by disrupting large sized alien translocations and promoting exchanges between crop and wild genomes.**

Abstract Resistance to mustard aphid (*Lipaphis erysimi*) infestation was significantly improved in *Brassica juncea* through *B. juncea-B. fruticulosa* introgression. However, linkage drag caused by introgressed chromatin fragments has so far prevented the deployment of this resistance source in commercial cultivars. We investigated the patterns of donor chromatin segment substitutions in the introgression lines (ILs) through genomic in situ hybridization (GISH) coupled with *B. juncea* chromosome-specific oligonucleotide probes. These allowed identification of large chromosome translocations from *B. fruticulosa* in the terminal regions of chromosomes A05, B02, B03 and B04 in three founder ILs (AD-64, 101 and 104). Only AD-101 carried an additional translocation at the sub-terminal to intercalary position in both homologues of chromosome A01. We validated these translocations with a reciprocal blast hit analysis using shotgun sequencing of three ILs and species-specific contigs/scaffolds (kb sized) from a de novo assembly of *B. fruticulosa*. Alien segment substitution on chromosome A05 could not be validated. Current studies also endeavoured to break linkage drag by exposing seeds to a heavy dose (200kR) of gamma radiation. Reduction in the size of introgressed chromatin fragments was observed in many M_3 plants. There was a complete loss of the alien chromosome fragment in one instance. A few M_3 plants with novel patterns of chromosome segment substitutions displayed improved agronomic performance coupled with resistance to mustard aphid. SNPs in such genomic spaces should aid the development of markers to track introgressed DNA and allow application in plant breeding.

Introduction

Mustard (*Brassica juncea* (L.) Czern & Coss.) is an important oilseed crop of dry areas in India, China, East Europe, Australia and Canada (Banuelos et al. 2013; Banga et al. 2015). It is an allotetraploid (AABB; $2n = 36$) that may have evolved through many independent hybridization events between *B. rapa* (AA; $2n = 20$) and *B. nigra* (BB; $2n = 16$) (Prakash et al. 2009; Kaur et al. 2014). Conflicting views exist regarding its geographic origin. Proposals include the Middle East (Olsson 1960; Vaughan et al. 1963; Axelsson et al. 2000; Prakash et al. 2009), Afghanistan (Vavilov 1949; Banga and Banga 2016) or the areas spanning Eastern Europe and China (Spect and Diederichsen 2001). Large morphotype and phenological diversity exist in this crop, possibly due to multiple events of domestication and human selected adaptations over wide eco-geographies (Banga and Banga 2016). Despite an enormous germplasm base, reliable sources of resistance are not available for many pests and diseases in the primary gene pool of this crop. Mustard aphid (*Lipaphis erysimi* Kalt.) is a major pest that causes significant yield losses in mustard ($\leq 70\%$) by direct phloem feeding or as a vector for many viral diseases (Bakhetia and

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Sandhu 1973; Bakhetia and Bindra 1977; Malik and Anand 1984; Rohilla et al. 1987; Kumar and Banga 2017). Aphids can colonize and spread quickly due to their exponential rate of reproduction and uncanny ability to manipulate plant defences (Kliebenstein et al. 2002; Ahuja et al. 2010). We have tried to explore alternate sources of genetic diversity available in wild crop relatives. A wild *Brassicaceae* species, *B. fruticulosa* (FF; $2n = 16$) or Mediterranean cabbage was found to be resistant to aphids *Brevicoryne brassicae* (Cole1994a, b; Ellis and Farrell 1995; Ellis et al. 2000) and *L. erysimi* (Kumar et al. 2011). Our group developed an interspecific hybrid between *B. fruticulosa* and *B. rapa*, with a fair degree of allopairing between F and A/B genomes (Chandra et al. 2004). This prompted us to initiate the transfer of *B. fruticulosa* resistance to Indian mustard (Kumar et al. 2011). The introgression strategy involved the use of synthetic allotetraploid (FFAA; $2n = 36$) as a pollen parent for hybridization with *B. juncea,* followed by one cycle of backcrossing with recipient *B. juncea*. Repeated selfing and selection for high pollen grain fertility in the back-cross progenies helped us to develop many *B. juncea- B. fruticulosa* introgression lines (ILs) with the euploid chromosome number of *B. juncea* (AABB; $2n = 4x = 36$). Subsequent analysis using molecular markers and genomic in situ hybridization (GISH) confirmed large alien chromosome segment substitutions in the host genome (Atri et al. 2012; Rana et al. 2017). These ILs also varied for their responses to mustard aphid infestation (Atri et al. 2012). Successful utilization of alien introgressions for cultivar development requires the introgressed variability to be heritable and transferable. Also, important is the linkage drag associated with the trait along with the possibility of its reduction through cycles of intermating, selfing and selection. This strategy requires significant levels of recombination between crop and wild genomes. However, the elimination of unwanted alleles can be problematic if the alien introgression is located in the recombination-repressed pericentromeric regions with low gene density (Wang et al. 2019a). Many population-genetic models predict greater differentiation over time in zones of low recombination due to the rapid sorting of ancestral alleles in the absence of gene flow (Nachman and Payseur 2012). The heritability of such translocated segments will be high if the repercussions in terms of gametic selection and reduced reproductive fitness are not limiting. Manipulation of pro- and anti-crossing over (CO) factors and the use of site-directed nucleases and epigenetic modifiers are recommended to increase the CO frequency and spread (Taagen et al. 2020).

The use of ionizing radiation to disrupt alien chromosome fragments is another possibility of reducing linkage drag. Compared to genome editing technologies, radiation-induced disruption of alien chromosome fragments is probably less efficient but a more breeder friendly option to reduce linkage drag as most plant breeders are more conversant with the use of ionizing radiations for crop improvement. Furthermore, application of genome editing technologies requires a prior knowledge of all gene sequences and their mode of action that confer linkage drag. It is technically challenging to identify and manipulate introgressed genes in a single experiment. Ionizing radiation has been used to induce chromosome breaks and promote non-homologous chromosome translocations in wheat (Friebe et al. 1996; Zhou and Xia 2005; Szakacs et al. 2010; Wang et al. 2019b), cotton (Wang et al. 2018), radish (Kaneko et al. 1992) and rapeseed (Primard-Brisset et al. 2005). Heavy doses of gamma rays can also induce deletion mutations (Ritter and Durante 2010; Malek et al. 2012). Broken chromosome ends are sticky and may rejoin to precipitate deletions or structural rearrangements. Practical use of radiation to disrupt adverse linkages requires *a priori* knowledge of the site of the alien segment substitutions and ability to detect induced structural rearrangements in the following generations. In situ hybridization techniques are used to recognize the substituted alien chromosome fragments in the host genome because these are efficient and reliable. One can also use the sequencing data to fine-tune cytogenetic inferences and facilitate marker-assisted detection of recombination between host and wild genomes at the site of introgression. For the present studies, we undertook detailed cytogenetic and molecular analysis of three secondary ILs (AD-64, AD-101 and AD-104) and their 35 M_3 progenies, developed following gamma irradiation. We selected these ILs for their resistance to mustard aphid over several cycles of selfing and selection under aided epiphytotic conditions. Multicolour FISH and shotgun genome sequence analysis were used to catalogue alien segment substitutions and their disruption following exposure to gamma rays. We screened M_3 progenies for aphid resistance in a net house. These procedures allowed us to identify a few M_3 progenies, which combined aphid resistance with improved agronomic performance. We also showed some reduction in the size of alien segment substitutions in selected M_3 plants.

Materials and methods

Plant materials

Three ILs, AD-64, AD-101 and AD-104 formed the founder germplasm. These ILs were previously selected for aphid resistance through several cycles of selection and inbreeding under aided epiphytotic conditions out of a large set of secondary *B. juncea-B. fruticulosa* ILs. The breeding strategy is available as in Fig. 1. We took 25 g seeds by the weight of each IL for exposure to gamma radiation at 200kR. M_1 plants obtained following irradiation of three ILs were raised to set seed in our summer nursery at Keylong (32.5710°

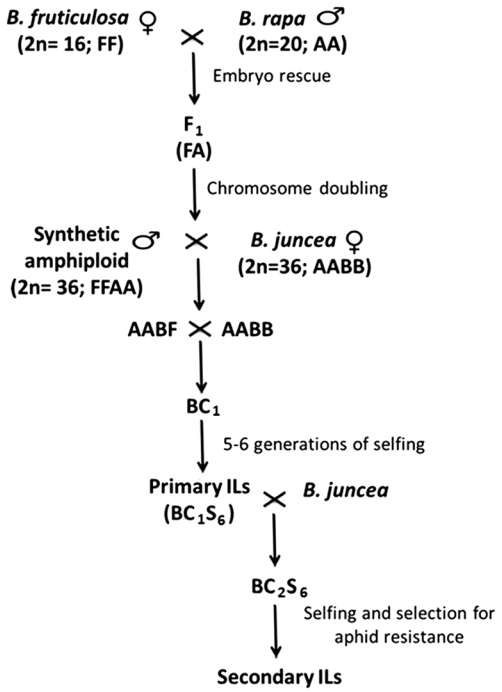

Fig. 1 Breeding scheme for the development and maintenance of *B. juncea- B. fruticulosa* introgression lines

N, 77.0320° E). M_2 seeds from M_1 plants, selected for low pollen grain stainability (< 50%) with 2% acetocarmine dye were selfed and harvested separately at maturity. M_1 plants with low pollen grain stainability were intentionally selected and selfed as they were expected to carry chromosome breakages or rearrangements induced by gamma irradiation. Low pollen grain stainability is normally associated with meiotic abnormalities. Fifty seeds from each identified M_1 plant were hand sown in five-metre-long rows. Over 160 plants (50 from AD-64 and 55 plants from each of AD-101 and AD-104) were selected in the M_2 generation on the basis of visibly detectable changes in plant morphology and phenology (e.g. earliness) and selfed to produce M_3 seeds.

Preparation of probes and blocking DNA

We isolated high-quality genomic DNA from *B. fruticulosa*, *B. nigra* and *B. juncea* cv. RLC-1, using Qiagen DNAeasy plant mini kit (REF 69,106, LOT 160,051,178). DNA extracted from *B. fruticulosa/ B. nigra* was sheared at high pressure for 2 min to obtain fragments between 500 and 1000bps. After that, the sheared DNA was slowly cooled to allow reannealing. We used commercially available nick-translation (Jena biosciences) or random primer labelling kits (Invitrogen) to directly label the DNA and develop in situ probes. These probes were later used along with available CentBr2 (Lim et al. 2005) and massive oligonucleotide pools (Agrawal et al. 2020) as probes. Briefly, the oligonucleotide pools (oligos 1, 2 and 3) comprised of several thousand oligonucleotides (47 mer) were designed from repeat-free different sections (sub-telomeric, intercalary, or sub-centromeric) of ten chromosomes of *B. rapa* using the methodology described in Agrawal et al. (2020). Simultaneous use of these pools as different colour probes in an in situ hybridization experiment produced a characteristic and reproducible binding pattern on every chromosome, which allowed their individual identification belonging to A, B, C and R genomes of Brassicaceae family (Agrawal et al. 2020). DNA from *B. juncea* was sheared at high pressure for 10 min, cooled rapidly and used as a block.

Preparation of chromosome spreads and in situhybridization

In situ hybridization experiments were conducted using a slight modification of the procedure reported by Schwarzacher and Heslop-Harrison (2000). We isolated the dividing meristematic cells from ILs by squashing root tips on glass slides for probe hybridization. The slides, used for in situ experiments, were first pretreated with RNAase and pepsin then re-fixed in 4% (w/v) para-formaldehyde. We omitted formamide from the hybridization mixture. By omitting formamide, we could improve the pace and efficiency of hybridization reaction (Jang and Weiss-Schneeweiss 2015). The probe mixture was applied to the marked area of slides and chromosomes were denatured in a thermal cycler at 75 °C for 7 min, then slowly cooled to 37 °C and kept overnight for two days in a humid chamber for hybridization. After hybridization, slides were washed at 42 °C for 2 min in 2×SSC buffer and 5 min in 0.1×SSC buffer (three times), respectively. Finally, chromosomes were counterstained with DAPI (4′, 6-diamidino-2-phenylindole), and fluorescence was visualized using an epifluorescence Zeiss microscope (Zeiss Metasystems AX-10). For reprobing of cells, immersion oil was carefully blotted off from the coverslip. The slides were kept at 37 °C for 5–10 min to reduce the viscosity of the glycerol/antifade mount and coverslips removed by lifting from the edge with a razor blade before washing three times in a Coplin jar filled with the detection buffer. Washing was initially done for 5 min and then repeated two times for 30 min each at room temperature. Slides were then incubated in 2×SSC twice for 5 min each. The slides were then dehydrated through an ethanol series (70%, 85% and 96% ethanol, 2 min each) and air-dried.

Phenotyping M_3 progenies of three ILs for aphid resistance

Fifty seeds each were harvested from 160 M_2 plants (50 plants from AD-64 and 55 each from AD-101 and AD-104) were planted in pots to raise 160 M_3 progenies at three pots

per progeny. The pots were placed in screen house well before initiation of flowering. M_3 progenies were screened for aphid infestation in insect-proof screen house along with three parental ILs and RLC-1 as a susceptible check in two replications during crop season, 2018–19. Aphids were artificially released three times at 40 aphids per plant/inoculation during early stages of flowering. Data were recorded at intervals of 15 days each for the aphid population after the last inoculation until complete pod formation on the main shoot. The number of aphids (nymphs and adults) was counted from top 10 cm, middle and lower portions of leaves from at least three plants from each M3 progeny/replication and averaged.

In silico identification of introgressions using shotgun genome sequence analysis

Leaves of all M_3 plants and parental ILs were collected, frozen in liquid nitrogen and stored at − 80 °C for DNA extraction. Qiagen DNAeasy plant mini kit was used to isolate genomic DNA from three ILs and 35 plants. These M_3 plants were selected on the basis of differences in their response to the aphid infestation. Shotgun sequencing of 3 ILs (20×) and 35 selected M_3 plants (5×) was outsourced to NxGenBio Life Sciences, New Delhi, India. High-quality DNA from each sample was used to complete the library construction. Selected libraries with appropriate insert sizes were then used for paired-end sequencing on Illumina HiSeq platform, with a read length of 150 bp at each end. The raw reads were processed to retain sequences of desired quality and adapter sequences were removed using the software Cutadapt (Martin 2011). Clean sequencing reads of three founder ILs and 35 M_3 plants were mapped to the *B. juncea* genome reference (Yang et al. 2016) using Bowtie2 (Langmead and Salzberg 2012) software within the Geneious®v11.1.5 (www.geneious.com) platform with default settings. Mapping of reads was followed by the generation of consensus FASTA sequence files for each IL for use in subsequent analysis. We followed a simple strategy to graphically represent alien segment substitutions in the ILs (Fig. 2). We used in-house de novo draft assembly of the aphid resistance donor species, *B. fruticulosa* for identifying chromosome segment substitutions in founder ILs and 35 M_3 plants. N50 of *B. fruticulosa* scaffolds was 483 bps with the largest scaffold of length more than 61 kb. The assembled *B. fruticulosa* scaffolds (2,710,391) were blasted against reference genome of *B. juncea*_V1.5 scaffolds with best hits showing more than 90% identity and more than 150 bps alignment length against *B. juncea* reference were removed from the core *B. fruticulosa* scaffolds. This approach of filtering the scaffolds was repeated successively with reference genome sequences of *B. rapa, B. nigra* and *B. oleracea* to

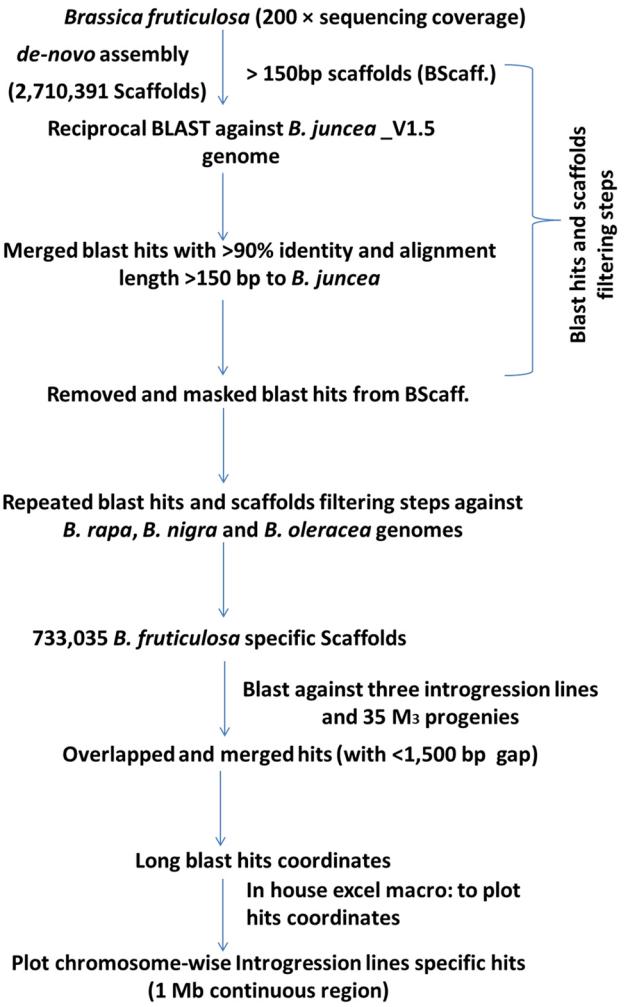

Fig. 2 Flowchart of the methodology used for identifying *B. fruticulosa* introgressions

retain only the *B. fruticulosa*-specific scaffolds. Only 27% of the filtered scaffolds were *B. fruticulosa* specific. These were blasted (as a query) against the shotgun sequences of ILs and progenies, mapped on *B. juncea* reference genome. Reciprocal blast hits displaying more than 90% identity and more than 200 bps alignment length were plotted, with an in-house excel macro**.** This macro allows simultaneous loading and plotting of reciprocal blast hit data from a large number of lines with a defined filtering criterion to score introgressions. We classified contiguous hits covering at least 1Mb region in a stretch as translocation/introgression. For plotting an alien segment substitution, two adjacent hits with a gap of < 1500 bps were considered as one hit. We also conducted phylogenetic analysis to compare the mapped genome sequences of three ILs and 35 M_3 plants with those from two *B. juncea* cvs. RLC-3 and PBR 357, using the software SKMER (Sarmashghi et al. 2019). The resultant distance matrix was visualized with split tree software (Huson

1998). SNP density (within 1 Mb window) was estimated and plotted by r-package "CMplot" (https://github.com/YinLiLin/CMplot).

Results

Molecular cytogenetic characterization of Introgression lines

A *B. fruticulosa* GISH probe was first used to detect alien chromosome segment substitutions in the ILs. The same slides were later reanalysed with centBr2, B-genome and massive oligonucleotide pool probes to identify chromosomes carrying alien segment substitutions. Chromosome 'band' patterns developed with oligonucleotide libraries along with centBr2- and *B. nigra*-specific probes allowed us to recognize eighteen *B. juncea* chromosomes in the test ILs. Of these, IL AD-101 revealed *B. fruticulosa*-specific DNA hybridization at the distal half of both homologues in chromosomes A05, B02, B03 and B04 (Fig. 3a, Fig. S1a, b, c). We also found the same DNA hybridization signal at sub-terminal to intercalary positions on chromosome A01. This segment substitution represented approximately 30% of the host chromosome arm. IL AD-64 and AD-104 also revealed *B. fruticulosa*-specific fluorescent signals on chromosomes A05, B02, B03 and B04 at positions analogous to those discovered for AD-101 (Fig. 3b, Fig. S1d, e, f). The results of in situhybridization of AD-104 were exactly same as those obtained for AD-64. Its image is not included in composite Fig. 3 to avoid loss in image quality. Euploid *B. juncea* was also used as a control in our experiments. It did not produce any signals from *B. fruticulosa* probe, thus suggesting specificity of the experiment (Fig. S2a, b).

In silico identification of alien chromosome fragments in three ILs

We used the whole genome shotgun sequencing data of three ILs to validate alien fragment substitutions visualized following in situ hybridization. The generated chromosome graphs re-confirmed the chromosome identities of three ILs harbouring alien fragment substitutions (Fig. 4). *B. fruticulosa*-specific fluorescent signal at the terminal position of chromosome A05 could not be confirmed through bioinformatic pipeline. Bioinformatic analysis was also suggestive of small alien segment substitutions, which could not be visualized through in situ studies. The maximum number of translocation breakpoints was detected in AD-101 (18) on four chromosomes (A01, B02, B03 and B04) followed by AD-64 (16) and AD-104 (13) involving three chromosomes (B02, B03 and B04) (Fig. 4, Table 1). The predicted size of alien segments ranged from 1 to 4Mb, and the largest being on B03 in IL AD-64. Marginally lower read alignment (Table S1) coupled with high SNP density (Fig. S3) was recorded at the introgression sites predicted through in silico analysis. However, decline in the read depth at the sites of introgression was not very obvious.

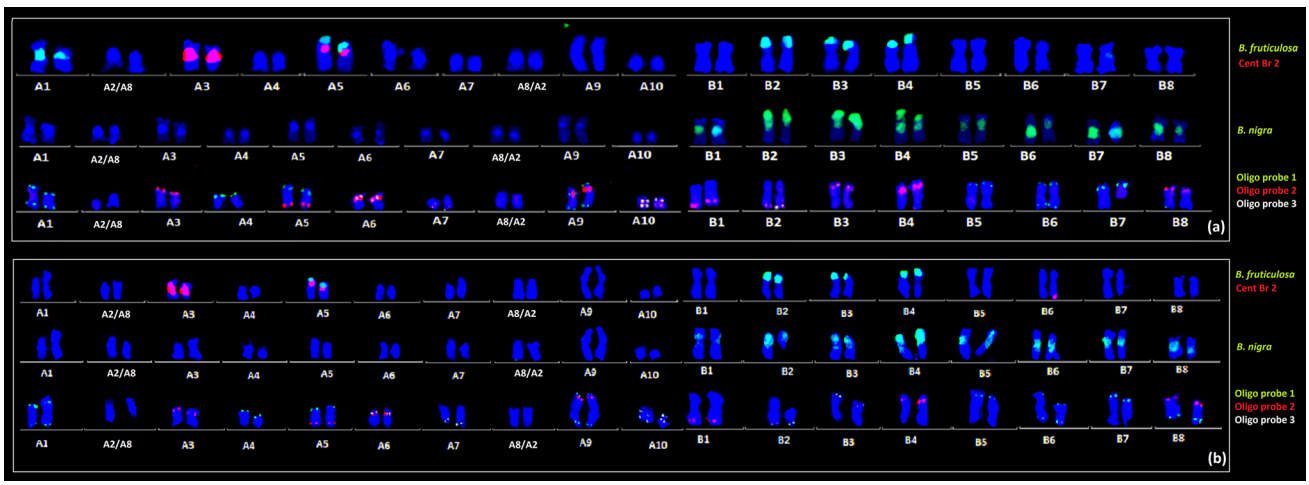

Fig. 3 Karyotypes of ILs AD-101(**a**) and AD-64 (**b**). These were developed through digital excision of identified chromosomes showing introgressed segments on chromosomes A01, A05, B02, B03 and B04 for AD-101 and chromosomes A05, B02, B03 and B04 for AD-64. AD 104 also showed exactly same introgression scenario as is depicted for AD-64

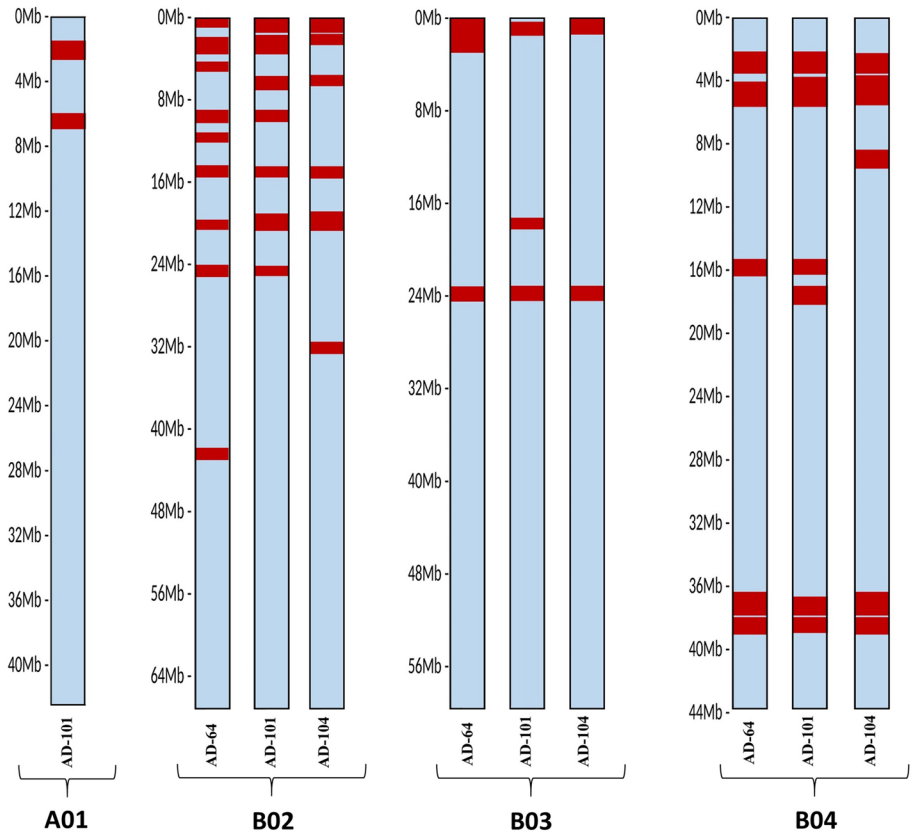

Fig. 4 Size and distribution of introgressed fragments on chromosomes A01, B02, B03 and B04 of three introgression lines

Resistance responses to mustard aphid in mutagenized progenies of parent ILs

Seed germination of around 40% was observed in the M_1 plants obtained following gamma irradiation. Most of these plants had normal morphology and pollen grain stainability, but some (10–15%) showed low-to-moderate levels of pollen grain stainability (Fig. 5a–d). M_2 progenies varied for plant height, leaf morphology and flowering time. We also noted certain morphological aberrations like leaf chlorosis during early stages of plant growth. 160 M_3 progenies along with three parental lines and one susceptible check were assessed for aphid resistance under artificial aided epiphytotic conditions in an insect-proof screen house. The founder ILs were consistent in their resistance responses to mustard aphid. Individual M_3 progenies, however, varied substantially for aphid infestation (Fig. 6a-e). Replication effects were non-significant (Table 2). Complete loss of resistance was observed in 40 progenies. The remaining progenies showed variable (moderate to high) resistance responses along with variations in flowering time, plant height and leaf morphology (Fig. 6a–e, Fig. 7a–f). Twelve progenies (N-6, N-23, N-25, N-31, N-36, N-38, N-61, N-70, N-73, N-116, N-140, N-144) showed resistance levels at-par with the founder ILs along with improved morphological features such as reduced plant height and early flowering.

In situ hybridization analysis of selected M_3 plants

We selected twelve M_3 plants—four per IL—for in situ studies. These were identified on the basis of their improved agronomic performance along with aphid resistance. We used the previously described cytogenetic toolkit (Agrawal et al. 2020) to observe segment loss, segment gain or induced chromosome exchanges. In situ analysis of eleven progenies revealed introgression patterns similar to those observed for founder ILs. One resistant progeny (N-140), derived from AD-104, showed the loss of introgressed segment from both homologues of chromosome A05 (Fig. 8). There was no corresponding loss of resistance to mustard aphid. The remaining plants failed to produce any changes in the fluorescent signal patterns noted for the founder ILs.

Analysis of the shotgun genome sequences of selected M_3 plants

Post-irradiation, disruptions of large *B. fruticulosa* segment substitutions in the mustard ILs were apparent from in silico analysis of the shotgun sequencing data of 35 M_3 plants (Fig. 9). These M_3 plants were selected to represent the entire spectrum of variation for defensive responses to

Table 1 Frequency and size of introgressed chromosome fragments in introgression lines and their variants following gamma irradiation

M_3 plants	No. of introgressed fragments					Total size (bps)	Decrease in size of fragments in M_3 progenies relative to ILS (%)	Response to aphid infestation
	B2	B3	B4	A1	Total			
AD- 64	9	2	5	–	16	20,011,401	—	Resistant
N-22	13	4	8	–	25	15,946,806	20.31	Moderately resistant
N-23	14	8	12	–	34	15,832,509	20.88	Resistant
N-25	15	8	11	–	34	16,214,424	18.97	Resistant
N-31	15	7	8	–	30	16,808,963	16.00	Resistant
N-33	15	8	8	–	31	15,499,599	22.55	Susceptible
N-36	16	8	8	–	32	15,442,729	22.83	Resistant
N-38	14	4	8	–	26	16,496,866	17.56	Resistant
N-39	15	5	9	–	29	15,729,815	21.40	Susceptible
N-42	12	4	7	–	23	17,106,077	14.52	Moderately resistant
N-65	21	8	17	–	46	10,352,982	48.26	Moderately resistant
N-67	14	4	8	–	26	16,783,395	16.13	Moderately resistant
N-70	13	6	7	–	26	16,080,302	19.64	Resistant
N-78	22	10	17	–	49	60,872,72	69.58	Moderately resistant
N-111	16	8	7	–	31	15,878,365	20.65	Susceptible
N-149	13	10	8	–	31	16,955,563	15.27	Susceptible
AD-101	7	3	6	2	18	21,438,705	–-	Resistant
N-6	14	7	14	3	38	16,571,440	22.70	Resistant
N-10	13	9	10	6	38	16,687,880	22.16	Susceptible
N-13	12	7	11	5	35	15,276,920	28.74	Moderately resistant
N-116	13	7	12	5	37	16,034,490	25.21	Resistant
N-143	11	6	9	5	31	17,303,112	19.29	Moderately resistant
N-144	13	7	11	6	37	15,891,381	25.88	Resistant
N-147	14	7	15	4	40	16,446,793	23.28	Moderately resistant
N-157	14	7	12	6	39	16,906,497	21.14	Susceptible
N-159	14	8	13	5	40	16,113,164	24.84	Moderately resistant
N-160	12	5	8	6	31	17,232,918	19.62	Moderately resistant
N-163	14	8	16	5	43	14,245,805	33.55	Moderately resistant
AD-104	6	2	5	–	13	16,286,964	–-	Resistant
N-46	16	6	10	–	32	11,782,792	27.66	Susceptible
N-47	13	5	10	–	27	11,997,595	26.34	Moderately resistant
N-48	11	5	8	–	24	12,499,285	23.26	Moderately resistant
N-61	11	5	10	–	26	13,578,093	16.63	Resistant
N-71	14	5	15	–	34	12,729,205	21.84	Moderately resistant
N-73	14	5	12	–	31	11,384,310	30.10	Resistant
N-89	14	4	9	–	27	12,548,518	22.95	Susceptible
N-140	16	6	9	–	31	12,450,968	23.55	Resistant
N-141	10	6	9	–	25	12,546,964	22.96	Moderately resistant

aphid infestation observed in M_3 progenies under aided epiphytotic conditions. In most of the cases, large chromosome introgressions were split (Table 1). Aphid-resistant progenies N-65, N-78 of AD-64; N-13, N-116, 144, N-163 of AD-101 and N-47, N-73 of AD-104 appeared important as their representative plants revealed a 25% reduction in the size of alien segment substitutions when compared with the founder ILs (Table 1). We also noted the emergence of some novel fragments on the chromosomes carrying alien segment substitutions (Table 3). These novel fragments varied both in number and size among the progenies of three ILs. Progeny N-159 of AD-101 showed maximum number (eight) of novel fragments followed by progenies N-144 and N-25 of AD-101 and AD-64, respectively.

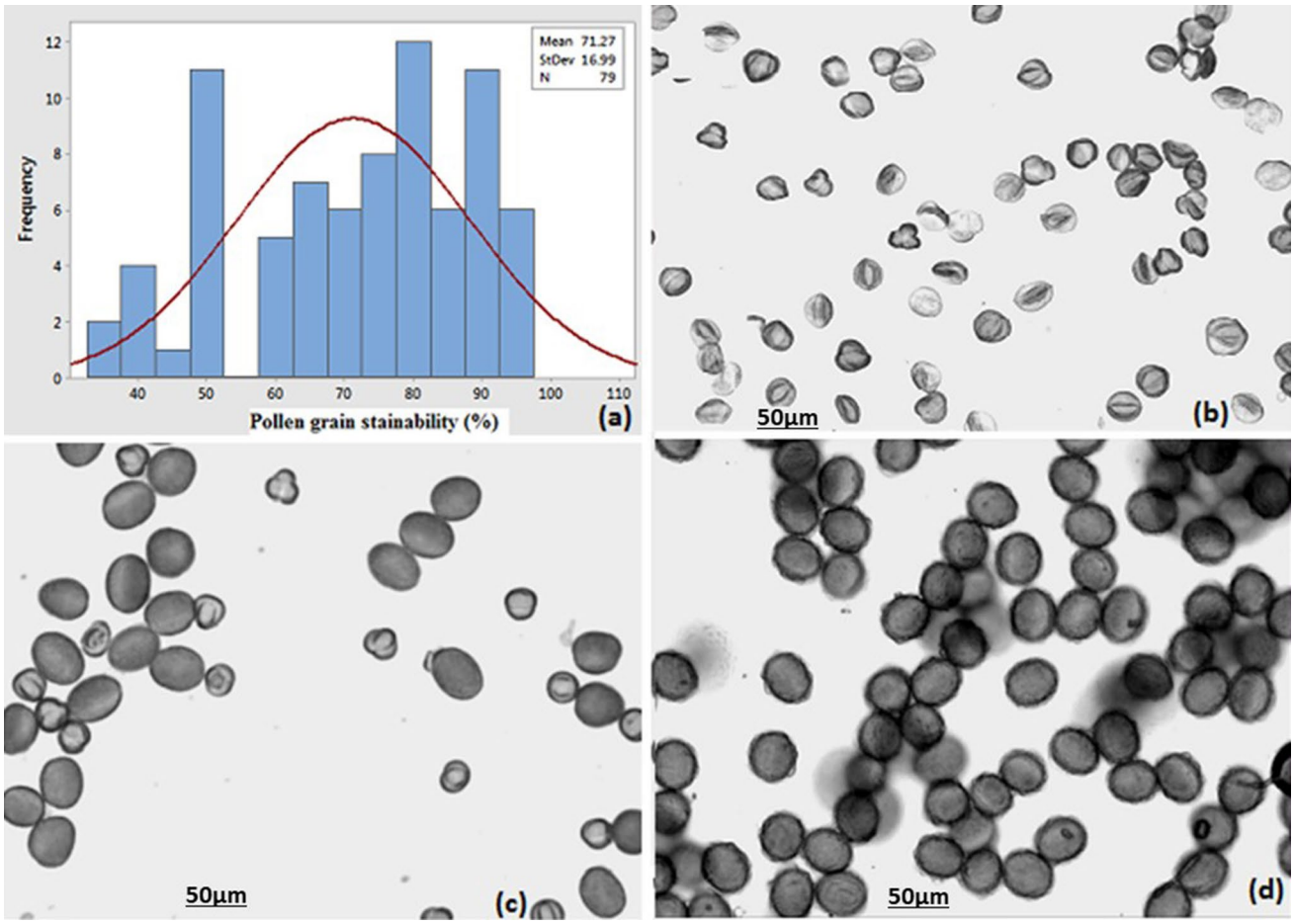

Fig. 5 **a** Frequency histogram showing variation in pollen grain stainability of M_2 progenies obtained following irradiation of three ILs, **b** no pollen grain stainability, **c** 50% pollen grain stainability and (**d**) 100% pollen grain stainability

Phylogenetic analysis

We also undertook genome-scale phylogenetic analysis of three founder ILs, plants representing 35 M_3 progenies and two *B. juncea* cvs PBR 357 and RLC-3. We also included reference genome assembly of *B. juncea*_V1.5 for comparison. Plants representing M_3 progenies with reduced introgression load veered close to *B. juncea* compared to their introgressed parents. The constructed phylogenetic tree divided test genotypes into two major groups, with some genotypes widely dispersed in the middle of two groupings (Fig. 10). The founder ILs were genetically distant from each other. M_3 plants representative of N-31, N-36, N-38 from AD-64 and N-144 from AD-101 showed increased homologies to *B. juncea* checks, while maintaining high aphid resistance. Another set of eight moderately aphid-resistant progenies (N-42, N-47, N-48, N143, N147, N-159, N160, N-163) also shared close genetic relatedness to *B. juncea* cultivars. Three progenies (N-47, N-144 and N-163) are of special interest for future investigations as these combined low introgressive load with high aphid resistance.

Discussion

Wide hybridization is widely used to transfer genes of interest from crop wild relatives into the crop species of interest. Genetic exchanges between the crop and wild genomes are expected if wide hybrids are at least partly fertile and there is some degree of allosyndetic pairing. However, allosyndetic pairing is either suppressed (as in wheat) or is restricted to small homoeologous stretches of chromosomes in most wide hybrids. Unequal chromosome pairing, breakages, random reunions of fragmented chromosomes coupled and impaired transmission frequency of translocated chromosomes are more common. These may lead to restructured chromosomes or chromosome number variations. Introgressions are deleterious if they affect reproductive performance of plants. However, these can be adaptive if they

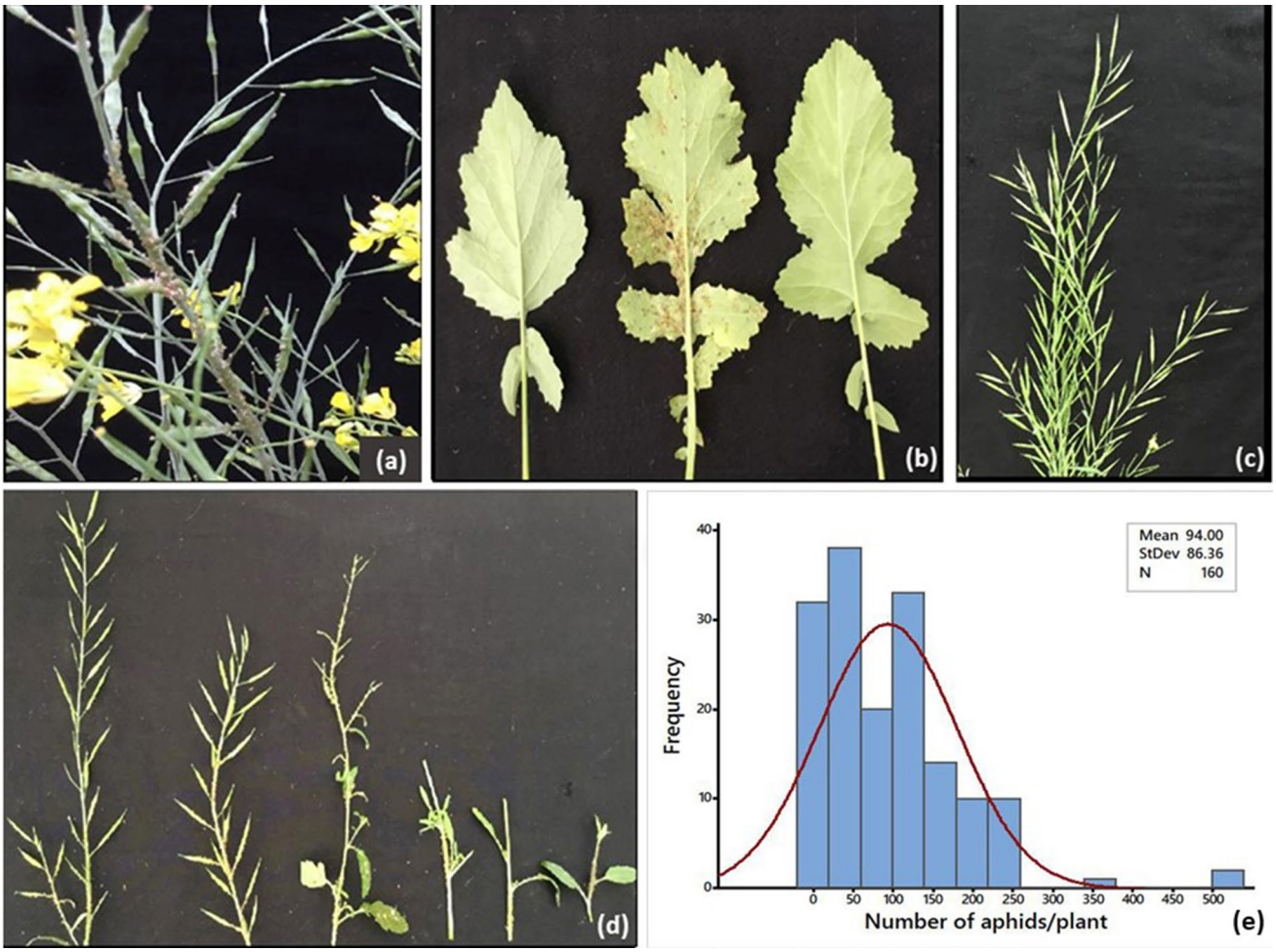

Fig. 6 Variation for aphid infestation in M_3 progenies. **a** High, **c** low and **d** varied aphid abundance on developing siliquae, leaf axils and **b** the lower surface of leaves. **e** Frequency histogram depicting variation in the mean count of aphid infested/plant in M_3 progenies

Table 2 Analysis of variance for aphid infestation in M_3 progenies

Source	DF	Sum of squares	Mean SS	F-value
Genotype	159	3,841,301	24,159.1	2766**
Replication	2	89	44.5	5.09
Error	318	2778	8.7	
Total	479	3,844,168		

**Significant at 1% level

improve allelic variation, which is subject to natural selection (Sachdeva and Barton 2018; Burgarella et al. 2019). Repeated cycles of natural selection contribute to improved reproductive competence and phenotypic plasticity of the recipient species by weeding out harmful genes (Whitney and Gabler 2008). Population-genetic studies and analysis of whole genome sequences have shown widespread occurrence of adaptive introgressions in many angiosperms (Hufford et al. 2013; Whitney et al. 2015; Arnold et al. 2016; Schmickl et al. 2017; Civáň and Brown 2018; Cheng et al. 2019; Santos et al. 2019). Exploiting wide hybridizations to improve crop traits is challenging as alien introgressions are often associated with linkage drag in the form of reduced fecundity and poor agronomic performance. Linkage drag is caused by the gain of unwanted genetic variation along with the genes of interest or the inability of alien segment substitutions to sufficiently compensate for the loss of the host genome (Gudi et al.2020). Linkage drag may also result from DNA methylation (Liu et al. 2004) or the unpredictable interactions between the two diverse transcriptional networks (Landry et al. 2007). Potential economic costs of long-winded introgressive breeding and its uncertain outcome are major deterrents for most plant breeders to engage in the pre-breeding researches. However, improved ability to overcome species barriers and trace introgressions has reignited interest in such researches (Gill et al. 2011; Rana et al. 2017, 2019; King et al. 2018; Atri et al. 2019; Katche et al. 2019; Gupta and Banga 2020; Hao et al. 2020).

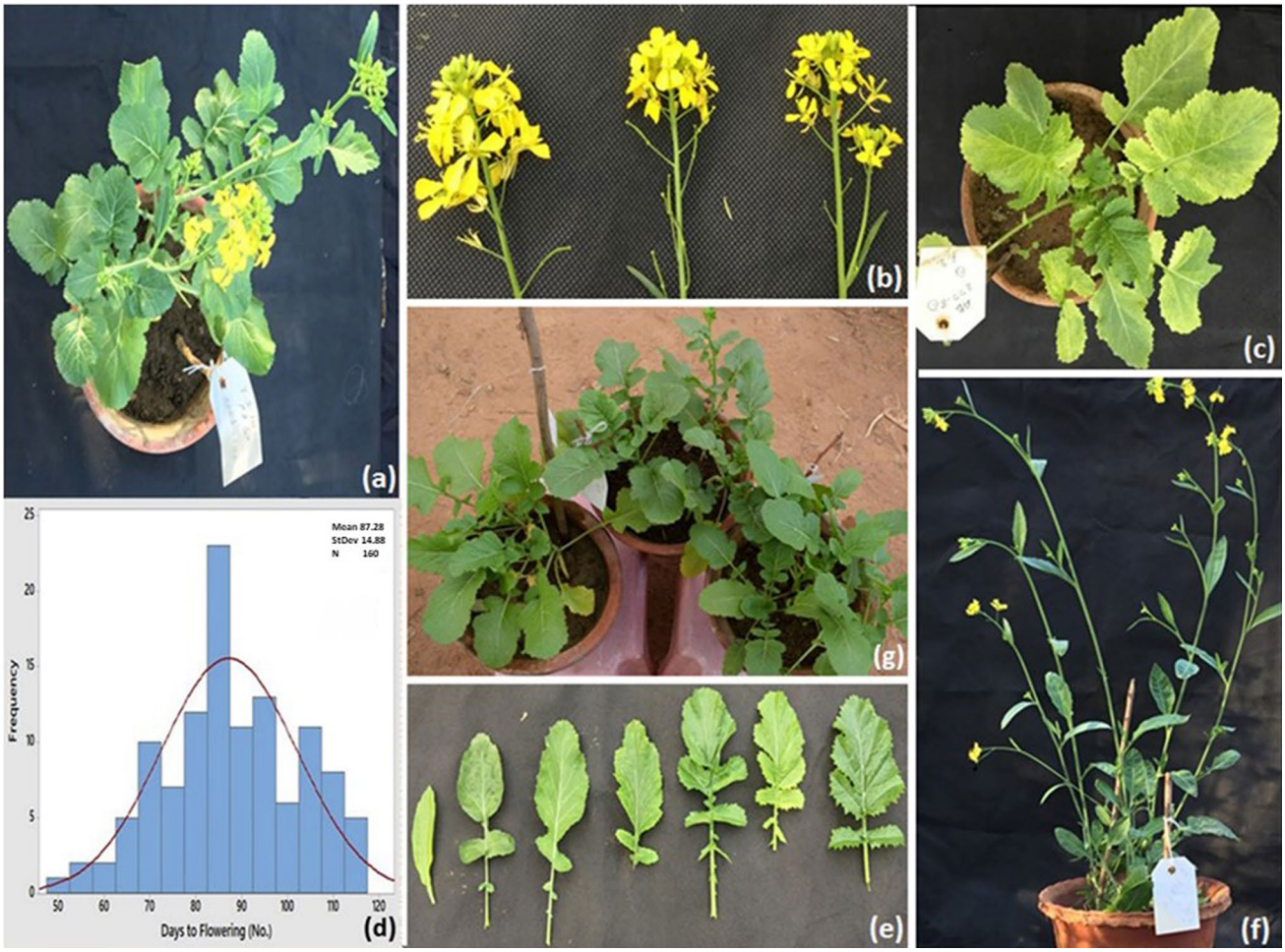

Fig. 7 Phenotypic variation recorded in M_3 generation for **a**, **c** leaf colour, **b** flower size, **d** flowering time, **e** leaf shape and size and **f** plant morphology

Crop brassicas comprise six economically important species. These arose from repeated cycles of polyploidy, diversification and domestication (Lysak et al. 2005). The secondary gene pool of crop brassicas constitutes massive reservoirs of nuclear and cytoplasmically encoded variation and many attempts have been made to transfer the genes of interest into cultivated forms (Prakash et al. 2009; Banga and Banga 2016; Gupta and Banga 2020). Being ancient polyploids, crop brassicas possess high genotypic plasticity and can tolerate heavy genetic load imposed by alien introgressions. We had previously reported the development of *B. juncea- B. fruticulosa* ILs, varying for resistance responses to mustard aphid (Kumar et al. 2011; Atri et al. 2012). Many of these ILs showed high field resistance to mustard aphid. However, linkage drag as reflected in poor agronomic value and lower seed set has so far prevented their use in mustard improvement programmes. Theoretically, this linkage drag can be reduced by minimizing the size of alien segment substitutions or by inducing a loss of function in unwanted genes.

Charting the landscape of alien introgression is an important first step for devising more targeted strategies for reducing linkage drag. In this communication, we report chromosome positions of introgressed *B. fruticulosa* chromosome fragments in three founder ILs by using genomic in situ hybridization (GISH) coupled with *Brassica* chromosome-specific massive oligo-pools (Agrawal et al. 2020). These allowed recognize segment substitutions on chromosomes A05, B02, B03 and B04 in AD-64, AD-101 and AD-104. AD-101 also harboured an additional *B. fruticulosa* fragment at the sub-terminal to the intercalary region of chromosome A01. Terminal translocations are more frequent as homoeologous exchanges are likely to occur more frequently in the telomeric regions (Lukaszewski 1995). GISH is the method of choice for analysing genome composition and intergenomic pairing.

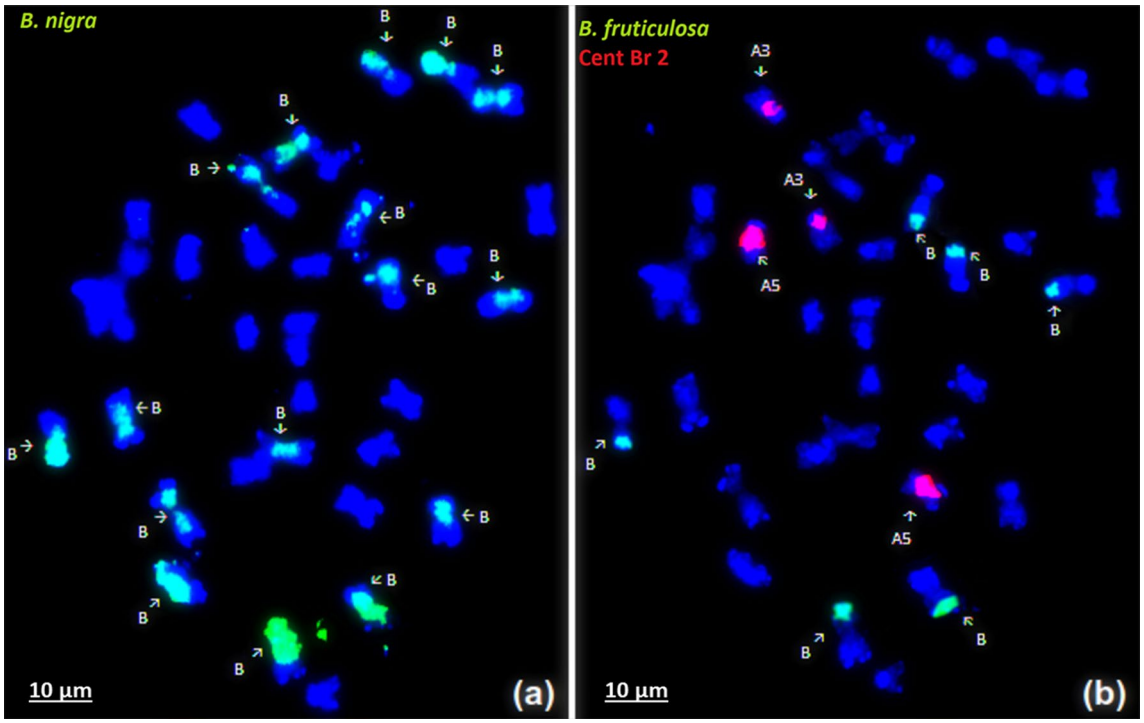

Fig. 8 Fluorescent in situ hybridization on mitotic chromosome spreads of M_3 progeny from IL-AD104 depicting loss of introgressed fragment from chromosome A05 using **a** *B. nigra* genomic DNA (green) and **b** *B. fruticulosa* genomic DNA (green) and Cent Br2 (red) (color figure online)

However, this technique can only visualize large sized segment substitutions (Dong et al. 2000; Schwarzacher 2003; Wei et al. 2003; Khrustaleva et al. 2005; Herrera et al. 2007; Chen et al. 2012; Ramzan et al. 2017). We also used shotgun sequencing of three founder ILs and the recipient parent to further refine the outcomes of in situ experiments. It was possible to confirm all the observed introgressions, barring the one visualized on chromosome A05. This may be due to the unrepresentative nature of the small *B. fruticulosa* genome assembly used for in silico analysis. A more representative assembly of the donor species should help trace many smaller introgressions, which cannot be visualized with GISH. The regions of introgression in our ILs were associated with high SNP density, which along with large segmental deletions have been construed as signals of inter-species introgression in wheat (Cheng et al. 2019). In silico analysis was also indicative of multiple small segment exchanges between *B. juncea* and *B. fruticulosa* chromosomes. B-genome chromosomes harboured many more exchanges, due to greater homoeology that exists between B-genome of *B. juncea* and F genome of *B. fruticulosa* as compared to the A-genome (Chandra et al.2004). *B. nigra*, the B-genome donor to *B. juncea*, and *B. fruticulosa* both belong to the same *Sinapis* lineage (Warwick and Black 1991). Furthermore, our breeding strategy of hybridizing synthetic allotetraploid (FFAA) with *B. juncea* (AABB) was designed to promote allopairing between chromosomes belonging to B and F genomes in the F_1 hybrid (AABB × FFAAABF).

Aphid-resistant founder ILs were tall and late flowering. These also produced small seeds and had poor productivity, which are possible reflections of the linkage drag due to uncontrolled introduction of deleterious genes along with those for aphid resistance. Linkage drag associated with physical linkages is difficult to be removed in case of low recombination between alien and crop genomes. Two cycles of backcrossing with diverse *B. juncea* genotypes and multiple rounds of selfing failed to produce any aphid-resistant IL with improved agronomic performance. Our cytogenetic and in silico studies revealed partial reduction in the size of alien segment substitutions in many M_3 plants. These confirmed the past reports that ionizing radiations can induce double-strand breaks (Preuss et al. 2000), InDels (Hase et al. 2018) and other structural alterations (Hosseinimehr 2010; Oladosu et al. 2015). Induced chromosome breaks and rejoining of aberrant ends can affect the size of segment substitutions by deletions, inversions or insertions (Shirley et al. 1992; Gorbunova and Levy 1999). Many of the M_3 progenies performed better as compared to the founder ILs. The improved agronomic performance may be associated with the observed reduction in size introgressed chromosome fragments along with induced mutations in the introgressed or host genomes

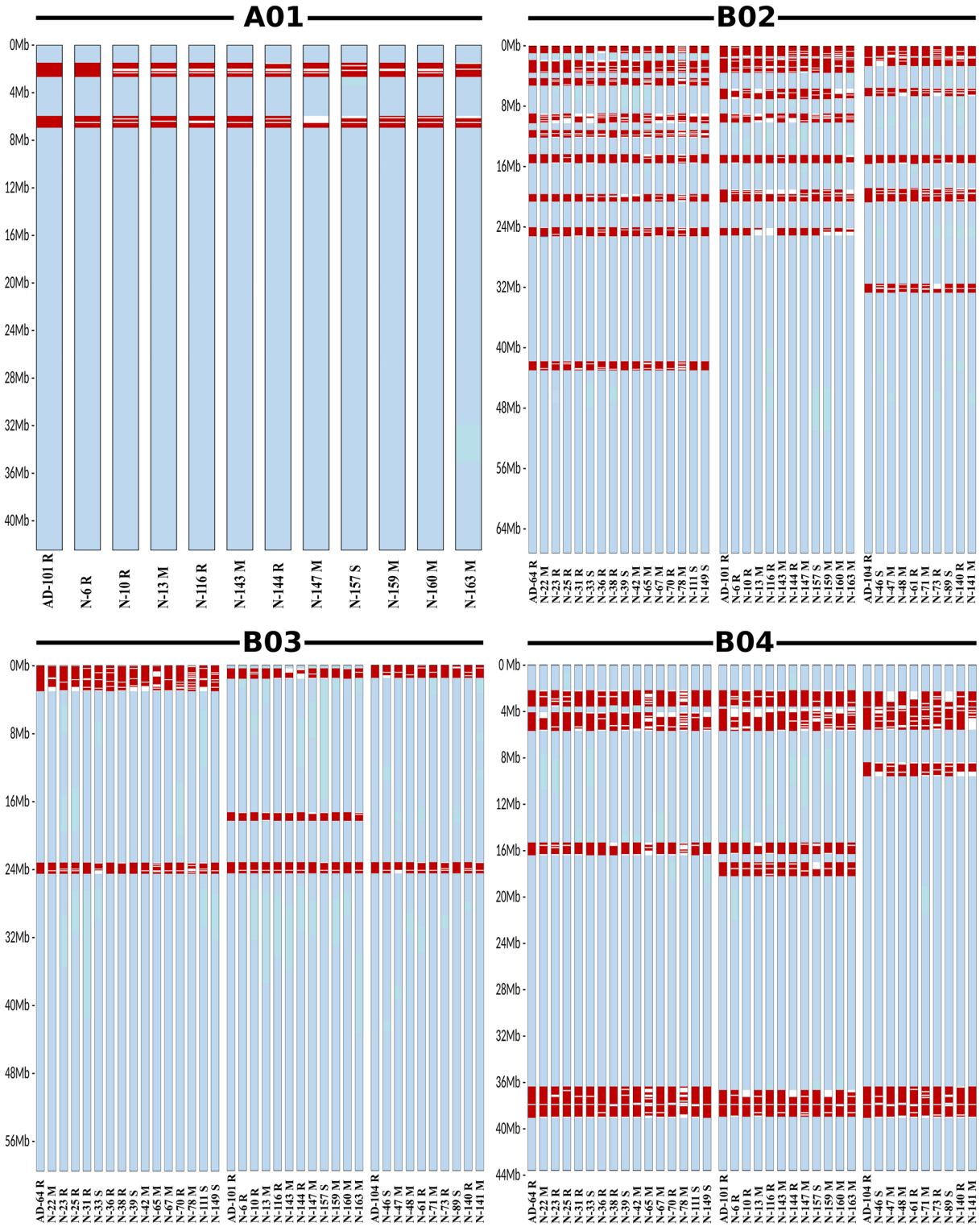

Fig. 9 Graphical image of chromosomes A01, B02, B03 and B04 of three introgression lines and selected M_3 plants (introgressions are shown in red, white background indicate breakdown) (color figure online)

(Kazama et al. 2017). Improved transmission frequency of the fertility restorer gene *Rfo* for *ogura* CMS has been reported following irradiation in *B. napus* (Primard-Brisset et al. 2005). Irradiation promote increased frequency of mutations, recombination (Larik et al. 2009) and chromosome breakages. Chromosome breakages generally produce

Table 3 Fragments gain in the M_3 plants following gamma irradiation of three secondary ILs

Genotype	No. of novel introgressed fragments				
	B2	B3	B4	A1	Total
AD- 64	0	0	0	–	0
N-22	1	0	1	–	2
N-23	2	3	1	–	6
N-25	2	3	2	–	7
N-31	3	2	1	–	6
N-33	2	2	2	–	6
N-36	1	0	0	–	1
N-38	2	0	0	–	2
N-39	2	0	1	–	3
N-42	1	0	2	–	3
N-65	4	0	0	–	4
N-67	1	0	1	–	2
N-70	1	1	1	–	3
N-78	0	0	0	–	0
N-111	1	2	0	–	3
N-149	2	2	2	–	6
AD-101	0	0	0	0	0
N-6	1	3	2	0	6
N-10	1	2	1	0	4
N-13	2	2	0	0	4
N-116	4	2	2	0	8
N-143	0	2	1	0	3
N-144	2	3	2	0	7
N-147	2	3	1	0	6
N-157	1	3	1	1	6
N-159	3	4	1	0	8
N-160	1	2	1	0	4
N-163	2	2	1	1	6
AD-104	0	0	0	–	0
N-46	3	2	0	–	5
N-47	2	2	0	–	4
N-48	0	1	0	–	1
N-61	3	2	0	–	5
N-71	1	1	1	–	3
N-73	3	1	0	–	4
N-89	4	1	0	–	5
N-140	4	1	0	–	5
N-141	2	3	0	–	5

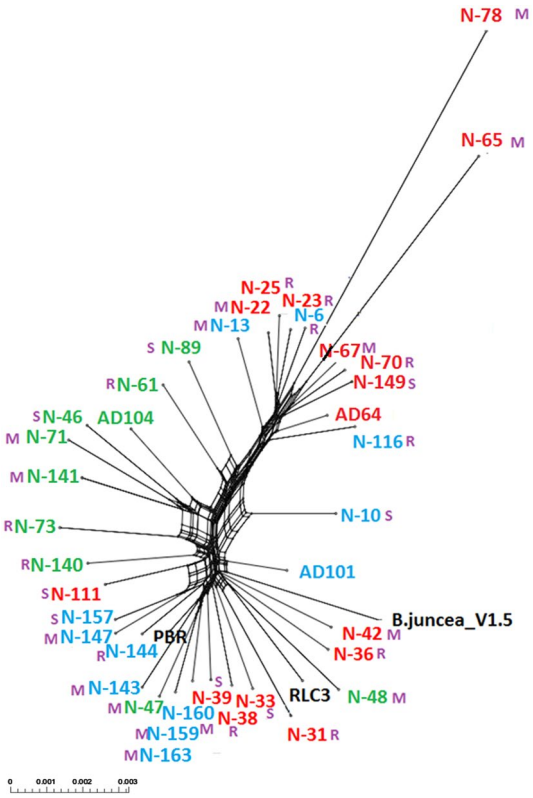

Fig. 10 Phylogenetic tree created from the consensus sequences of test M_3 progenies. M_3 progenies N-31, N-36, N-38 of AD-64 and N-144 of AD-101 showed increased homologies to *B. juncea* checks. The webbing effect in the tree indicates network block represents confidence sets

fragments with sticky ends, which facilitate chiasmata formation even between non-homologous chromosomes. We can also modulate recombination by using multiple recipient parents as the frequency of crossovers is influenced both by the karyotype and the genotype of the plants undergoing meiosis (Nicolas et al. 2008). The identification of five M_3 progenies (N-31, N-36, N-38, N-78 and N-65), all derived from AD-64, was the most significant outcome of our studies. These ILs combined higher aphid resistance with improved agronomic performance. N-31, N-36 and N-38 were also phylogenetically closer to cultivated *B. juncea* compared to AD-64. Complete loss of any introgressed fragment was rare and it was recorded only for the translocation located on chromosome A05 (N-140). For others, we could only detect small intercalary deletions in alien segment substitutions. Loss of aphid resistance in many M_3 progenies may have resulted from the partial loss of introgressed segment substitutions, deletions or loss of function mutations. The gain of novel fragments was also noted in a few M_3 plants. Such fragments can arise from double-stranded breaks and random reunion of chromosome fragments arising from the host and wild genomes. As pollen grain fertility is a reflection of meiotic stability, our strategy of selecting plants with low-to medium pollen grain fertility in M_1 and M_2 generations was expected to help in retaining plants with chromosome structural alterations. Two generations of selfing ($M_1M_2M_3$) facilitated the retention of several structural rearrangements as chromosome homozygotes, besides providing multiple targets for crossover formation between the translocated fragments and the host genome. Resultant

genomic mosaics can be further improved by repeated cycles of crossing and recombination.

Our inferences regarding radiation-induced disruption in the introgressed fragments are important as these reconfirm the practicality of irradiations to reduce or eliminate unwanted variation from the introgression lines. The use of molecular markers based on SNPs in the regions of alien segment substitutions can further improve breeding efficiencies by precise tracking of introgressed chromosome fragments and their breakpoints. The recombinant ILs reported in our studies will further the development of aphid-resistant mustard cultivars with improved agronomic performance.

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Author contribution statement S.S.B., N.A. and P.H.H. designed the research. S.S.B. and C.A. developed and maintained the introgression lines. N.A. and M.G. conducted cytogenetic studies. J.A. and N.A. carried out bioinformatics analysis. N.A and M.G interpreted the data and wrote the paper. S.S.B. and P.H.H. edited the manuscript and supervised the studies. All authors have read and approved this version of manuscript.

Availability of data and material Raw reads from sequencing data of the founder ILs are available at National Centre for Biotechnology Information as Bio-Project PRJNA662836 with bio sample IDs as SAMN18236321-23. Supply of plant materials will require prior approval from Biodiversity Authority of India.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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