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Isolation, genetic mapping and expression studies of a peptide deformylase gene, *DEF2*, from bold-seeded and small-seeded varieties of the oilseed crop, *Brassica juncea* (Indian mustard) and analysis of its over-expression and down-regulation on seed size of transgenic plants

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Abstract

B. juncea (Indian mustard) is an important oilseed crop with seed size or seed weight being a major contributor to crop yield. The nuclear-encoded *DEF2* gene produces a peptide deformylase that is targeted to the chloroplast. It is an essential enzyme with a role in embryo development, chloroplast function and photosynthesis. In this study, we analyzed the role of *DEF2* gene in modulation of seed size in *B. juncea* utilizing four germplasm lines, including two bold-seeded Indian varieties, Varuna and Pusa Bold, and two small-seeded east-European varieties, EH2 and Heera. Isolation of *DEF2* sequences from Varuna and Heera revealed the presence of two homologs of which, one homolog showed significantly higher expression levels during seed development stages in the bold-seeded varieties as compared to the small-seeded varieties. Genetic mapping studies based on polymorphisms between corresponding homologs identified one homolog co-localizing with a QTL for seed size in *B. juncea*. These observations indicated a possible correlation between the *DEF2* gene and seed size phenotype. To validate the functional effects of *DEF2* on seed size, transgenic plants were developed for antisense-mediated down-regulation of the *DEF2* gene in Varuna and over-expression of the gene in EH2. While 35 out of 37 single-locus antisense transgenic lines in Varuna showed decreased seed size, 11 out of 17 single-locus EH2 transgenic plants over-expressing the *DEF2* gene showed increased 100-seed weight of T1 seeds compared to the corresponding untransformed (control) plants. Homozygous lines were developed for a promising single-locus EH2 transgenic plant and analyzed for traits viz., seed size, oil content, fatty acid composition, protein content and seed yield per plant. Significant increase in seed size was obtained for the *DEF2* over-expression line in comparison to the untransformed control plants. The EH2 transgenic line with increased seed size generated in this study would be useful as a combiner for use in transgenic-based heterosis breeding programs in Indian mustard.

Keywords: seed size, *DEF2*, *Brassica juncea*, transgenics

Introduction

The cultivated *Brassica* genus comprises six inter-related species of which, the most important oilseed and vegetable crops are *B. rapa, B. oleracea*, *B. napus*, and *B. juncea*. *B. juncea* or Indian mustard (AABB genome) is an allopolyploid of *B. nigra* (BB genome) and *B. rapa* (AA genome) and is one of the most important oilseed crops of the Indian subcontinent. It is grown in more than 6.7 million ha of land in India during the winter season (October – March). The global germplasm diversity of *B. juncea* is characterized by two main gene pools, the Indian gene pool (comprising large- or bold-seeded varieties) and the East European gene pool (consisting of small-seeded varieties) (Kang et al., 2021). One of the frequently used parameters for measurement of seed size is thousand seed weight (TSW). Indian varieties such as Varuna and Pusa Bold are characterized by higher TSW (5.2±1.0 g) than east European varieties such as Heera and EH2 (2.3±0.4g) (Ramchiary et al., 2007). Earlier studies had identified heterotic combiners from the two gene pools (Pradhan et al., 1993; Srivastava et al., 2001). Based on these studies, DMH-1 (Pusa Bold × EH2), the first commercial hybrid of *B. juncea* released in India, yields ~30% more than the national check variety, Varuna (Sodhi et al., 2006) but has smaller seed size as compared to Indian varieties such as Varuna and Pusa Bold. The smaller seed size of the hybrid (TSW: <“ 3.9 g) is attributed to the East European parent, EH2, which, despite its small seeds, exhibits strong combining ability and leads to significant heterosis when crossed with the Indian variety, Varuna (Sodhi et al., 2006). Seed size is an important economic criterion for consumer preference. In conjunction with traits such as siliqua per plant and seeds per siliqua, it is also known to influence crop yield in mustard. Therefore, understanding seed size regulation and its improvement can contribute towards increasing crop yield.

The mechanisms underlying seed size regulation have been extensively studied in *Arabidopsis thaliana* and *Oryza sativa*. Seed size is known to be influenced by maternally imprinted genes, the ubiquitin–proteasome pathway, *IKU* pathway, mitogen-activated protein kinase (MAPK) signalling, phytohormones, G-protein signalling and transcriptional factors (Li et al., 2016; Savadi et al., 2018; Li et al., 2019). Wang et al. (2010) reported the influence of interactions between embryo size and endosperm, and integument proliferation and elongation on seed size. The transcription factor, *HAIKU (IKU)* was shown to affect seed size by regulating development of endosperm (Garcia et al., 2005). *MINI3*, *IKU1*, *IKU2* required in same pathway to expand and induce endosperm size (Luo et al., 2005). *SHB1* increase embryo cell expansion and proliferation (Zhou et al., 2009). Therefore, transcription factors that express during initial stages of seed development are majorly responsible for final seed size determination (Ruan et al., 2012; Du et al., 2017).

Several reports have described the effects of over-expression or down-regulation of different genes on seed size in *Brassica napus*. These include overexpression of *AtDA1R358K* through down-regulation of *BnDA1*, overexpression of *BnaA9.CKX2*, mutation of *BnaEOD1* homologs, down-regulation of *BnAP2* and reduced expression of *BnaUPL3.C03* (leading to increased LEC2 protein levels) which result in larger seeds in *B. napus* (Yan et al., 2012; Wang et al., 2017; Miller et al., 2019; Gu et al., 2023; Yan et al., 2023).

The timing of cellularization in the syncytial endosperm is recognized as a critical factor in determining seed size (Zhou et al., 2009; Wang et al., 2010). Additionally, early mitotic activity during initial stages of seed development, effective partitioning of carbohydrates and source and sink yield limitations are essential as they directly influence seed filling (Borrás et al., 2004; Ruan et al., 2012). *DEF2* is a nuclear gene encoding a peptide deformylase (PDF), which is targeted to the chloroplast (Dirk et al., 2001, 2008). Peptide deformylases are required for the maturation of chloroplast-translated proteins and are therefore, critical for chloroplast function and photosynthesis (Dirk et al., 2001). We hypothesized that overexpression of *DEF2* could potentially contribute towards enhanced seed size in *B. juncea*. In the present study, two large-seeded Indian varieties (Varuna and Pusa Bold) and two small-seeded East European Varieties (Heera and EH2) were analysed for variations in *DEF2* gene expression during seed development. Subsequently, the potential functional effects of *DEF2* on seed size were studied by development of knock-down and over-expression transgenic lines in Varuna and EH2 varieties, respectively.

Materials and methods

Plant material

Four *B. juncea* varieties namely, Varuna, Pusa Bold, EH2 and Heera were grown at the Delhi University Research Farm, Bawana Road, Delhi (28°44'40.1"N 77°07'30.8"E) during the crop growing season (October – March). The germplasm lines were maintained by self-pollination. Transgenic plants were grown in a containment net-house at the above location according to guidelines of the Department of Biotechnology, Government of India. In the growing season, the soil was treated with 1 ml l^{-1} Aldrin (pesticide) and 1 g l^{-1} Ridomil (fungicide) prior to sowing or transfer of plants. To ensure optimal plant growth, a row-to-row distance of 40 cm and a plant-to-plant distance of 25 cm in each row were maintained.

Isolation and genetic mapping of DEF2 gene

Full length *DEF2* gene sequences were isolated from the bold-seeded Indian variety, Varuna and small-seeded East European variety, Heera by PCR. Primer sequences and PCR conditions are provided in Supplementary Table 1. Following sequencing of the amplified products, corresponding homologs showing sequence polymorphism were mapped on a *B. juncea* linkage map generated using an F1 doubled haploid mapping population of 123 plants developed using the varieties, Varuna and Heera as parental lines. Genotyping of the F1DH population for *DEF2* homologs was done by allele-specific PCR using SNP-based primers that were designed for Varuna-specific and Heera-specific sequences by incorporating the variant nucleotide/s at the 3' end of the primers. The linkage map was generated using Joinmap version 2.0, using Kosambi's mapping function with a recombination threshold of 0.45 and a LOD score of 1.0.

Analysis of DEF2 transcript levels in bold-seeded and small-seeded germplasm lines

Controlled self-pollination was performed on unopened flower buds for each of the four varieties to analyse expression levels of *DEF2* at different stages of seed

development. Developing siliqua were harvested at intervals of 5, 10, 15, 20, 25, and 30 days after pollination (DAP). Total RNA was isolated using RNAqueous-4PCR kit from Ambion (USA) following manufacturer's instructions. cDNA synthesis was performed using high-capacity cDNA reverse transcription kit (Applied Biosystems, USA). Quantitative Real-time PCR experiments were performed on CFX Connect Real-Time PCR Detection system (Bio-Rad, USA) using SYBR-Green method. *ACT7* (Actin 7, AT5G09810) was used as an endogenous control as it was stably expressed and its expression did not vary significantly across seed developmental stages. PCR conditions for qRT-PCR were as follows: 95ºC for 10 minutes, 40 cycles of 15 seconds at 95ºC and 60ºC for 60 seconds. Two biological replicates each with two technical replicates were used for every sample.

Development of transformation vectors

The transgene expression cassette comprising of the *DEF2* gene in sense orientation (for over-expression) or antisense orientation (for knock-down) under transcriptional control of the *napin* seed-specific promoter and *ocspA* terminator was assembled by SOE-ing reactions (Horton, 1995; Vallejo et al., 1994). To assemble the cassette, the napin promoter was amplified from pPCRscript-*napin* plasmid *DEF2* gene was amplified from pGEM-T-*DEF2* plasmid, and the ocspA terminator was amplified from pSK+ocspA using appropriate SOE-ing PCR primers (Supplementary Table 2). Separate primers were used for development of the over-expression (OE) and antisense (AS) constructs. These amplified fragments were assembled using sequential SOEing-PCR reactions (Supplementary Table 2) to generate the napin-*DEF2OE*-ocspA and napin-*DEF2AS*-ocspA cassettes, which were cloned into pGEM-T easy vector and sequenced to check the fidelity of amplification and assembly. Following sequence verification, the complete cassette(s) from pGEM-T: napin-*DEF2(OE/AS)*-ocspA was excised as a *Not* I fragment, treated with Klenow DNA polymerase I to create blunt ends and cloned into the binary vector pPZP200-loxP-35Sde-*bar*-ocspA-loxP at *Swa I* site to generate the final over-expression (*DEF2OE*) and knock-down (*DEF2AS*) transformation vectors. The herbicide resistance-conferring *bar* gene, expressed using the CaMV35S double enhancer promoter (35Sde), was used as the selection marker in both constructs. The selection marker was placed between loxP sites to allow its subsequent removal using the Cre/lox system. The two transformation vectors were mobilized independently into *Agrobacterium tumefaciens* strain GV3101 by electroporation (ECM 399 Electroporation System) using standard procedures (Mattanovich et al., 1989).

Agrobacterium-mediated transformation of B. juncea

Agrobacterium-mediated transformation experiments on *B. juncea* cv. Varuna using the *DEF2AS* construct were performed using the standardized protocol reported earlier (Jagannath et al., 2003). For transformation of the recalcitrant small-seeded *B. juncea* variety, EH2 with the *DEF2OE* construct, a modified regeneration and transformation protocol was used as described below. Hypocotyls from 5 days-old seedlings were cut into 5-7 mm explants and precultured on MS-SIM3A liquid medium [MS media with 2,4-

D (0.05 mg l^{-1}), BAP (3 mg l^{-1}) and 2% glucose] for 18 hrs with mild shaking at 100 rpm. A single colony of *A. tumefaciens* GV3101 strain with the binary vector was inoculated in 5 ml YEB medium containing rifampicin (50 mg l^{-1}), gentamycin (20 mg l^{-1}) and spectinomycin (50 mg l^{-1}) and grown for 48 hours in an incubator shaker (250 rpm, 28ºC in dark). 250 µl of the above culture was re-inoculated in 30 ml of YEB (without antibiotics) and allowed to grow till it reached an OD600 of 0.6. The *Agrobacterium* suspension was pelleted by centrifugation at 5000 rpm for 10 minutes at 22ºC and re-suspended in liquid MS medium to a final OD600 of 0.3. Hypocotyl explants were infected with the above *Agrobacterium* suspension for 30 minutes in dark. The *Agrobacterium* suspension was removed after 30 minutes and explants were co-cultivated in liquid MS – SIM3A medium [MS media with 2,4-D (0.05 mg l^{-1}), BAP (3 mg l^{-1}) and 2% glucose] for 18-20 hrs, with mild shaking at 110 rpm at 23±1ºC. Co-cultivation was stopped by washing the explants with liquid MS-SIM3A medium containing augmentin (200 mg l^{-1}; active component Amoxicillin; GlaxoSmithKline, India). Explants were washed thrice with augmentin-containing medium (once for one minute and twice for 30 minutes each) and plated on solid MS-SIM3B medium containing 0.05 mg l^{-1} 2,4-D, 3 mg l^{-1} BAP, 20 µM silver nitrate, 200 mg l^{-1} augmentin, 2% glucose and 5 mg l^{-1} Basta (Agrevo, active component: phosphinothricin). Transformed green calli obtained were excised after 25-30 days and transferred to MS-SIM5 medium containing 0.015 mg l^{-1} 2,4-D, 1.5 mgl^{-1} BAP, 20 µM silver nitrate, 200 mg l^{-1} augmentin, 2% glucose and 5 mg l^{-1} Basta. Well-differentiated shoots were obtained after 25-35 days and transferred to rooting media (MS media with IBA 2 mg l^{-1}), Basta (5 mg l^{-1}) and augmentin (200 mg l^{-1}). Rooting took place in 15-20 days, following which the plantlets were maintained as nodal cultures on MS medium containing IBA (2 mg l^{-1}) and Basta (5 mg l^{-1}) until transplantation to a containment net-house during the growing season.

Identification of single locus events by segregation analysis, molecular confirmation and development of homozygous lines

To identify single locus events, fifty T_1 seeds from each T_0 plant were germinated in a 1:1 mixture of autoclaved soil and soilrite. The herbicide, Basta was sprayed (200 mg l^{-1}) after 15 days of growth and plants were scored for resistance or susceptibility. Single locus transgenic plants were identified based on segregation of Basta-resistant and sensitive plants in a 3:1 ratio in the T_1 progeny. Chi-square analysis was performed to determine the goodness of fit. Chi-square values <3.84 were considered as single locus lines. Molecular confirmation to validate the presence of the passenger gene in the Basta-resistant transgenic plants was performed by PCR using napin promoter-specific forward primer and *DEF2* gene-specific reverse primer (Supplementary Table 2). Amplification conditions were as follows: 94ºC for 5 minutes for initial denaturation followed by 30 cycles of amplification at 94ºC for 30 seconds, 57ºC for 30 seconds, 72ºC for 1 minute and a final extension at 72ºC for 5 minutes. T_1 progeny of selected single-locus transgenic lines showing desirable phenotype were self-pollinated and advanced to the T_2 generation to obtain homozygous lines. A minimum of 100 T_2 seeds derived from selected single-locus T_1 plants were sown in a containment net-house and sprayed with

Basta after fifteen days of sowing. Plants showing Basta resistance in 100% of T_2 progeny were identified as homozygous lines. Molecular confirmation for presence of the *DEF2* transgene in Basta-resistant plants was done by PCR as described earlier.

Measurement of seed traits: seed size, oil content, fatty acid composition and total protein

T_1 seeds obtained by self-pollination of independent T_0 transgenic plants were analysed for seed size by measurement of hundred seed weight (HSW). Homozygous lines were analysed for seed size by different approaches using open-pollinated seeds with at least three biological replicates. The first approach involved measurement of the weight (in grams) of yield per plant and 1000 seeds [thousand seed weight (TSW)]. In another approach, fifty seeds were arranged on a graph paper in a straight line and the occupied length was recorded in centimetres. The third approach involved counting the number of seeds that could be accommodated in one cm^2 grid of a graph paper. Measurement of seed oil content, fatty acid composition and protein content was done with five

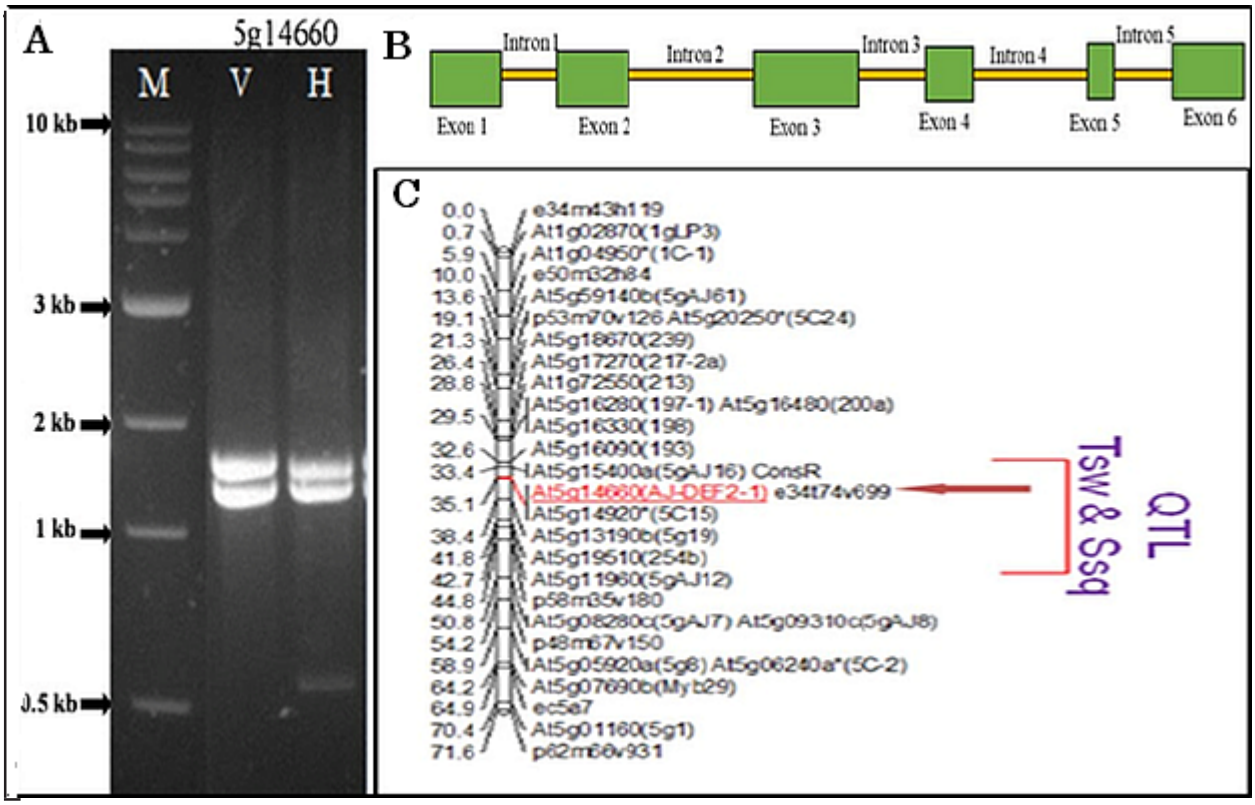

Fig. 1. (A) PCR amplification of full-length *DEF2* gene. Two homologs each were amplified from bold-seeded Indian variety, Varuna and small-seeded East European variety, Heera. Lane M: NEB 1 Kb ladder; Lane V: Amplification in bold-seeded Indian variety, Varuna, Lane H: Amplification in small-seeded East-European variety, Heera (B) Structural organization of *DEF2* gene in *B. juncea* var. Varuna (C) Co-localization of mapped homolog 'a' of *DEF2* gene with TSW seed size and seed per siliqua QTL on A10 linkage group.

biological replicates using Near Infra-Red Spectroscopy (NIRS) at CGMCP, University of Delhi South Campus. Students' *t*-test was performed to assess the significance of recorded variations for all the analysed traits.

Results

Isolation and structural characterization of DEF2 gene from bold-seeded and small-seeded varieties of B. juncea

In the current study, based on a genome-wide synteny map between Arabidopsis and *B. juncea* we observed that orthologs of the Arabidopsis *DEF2* gene (At5g14660) co-localize with a major seed size QTL on the A10 linkage group of *B. juncea*. Since *B. juncea* is an allopolyploid, it is expected to harbour multiple homologs (paralogs and/or orthologs) of the *DEF2* gene. Full-length sequences of the *DEF2* gene were amplified from genomic DNA of Varuna and Heera using primers designed from conserved sequences between *Arabidopsis* and *Brassica* species, with a preference for sequences from *B. rapa* and *B. nigra* (progenitors of *B. juncea*). We detected two fragments (designated 'a' and 'b') on agarose gel electrophoresis which potentially represent two homologs of the gene in *B. juncea* (Fig. 1 A). Following sequencing of the amplified fragments, the full-length genes were characterized for their intron/exon structure which predicted six exons and five introns in the *DEF2* gene of *B. juncea* (Fig. 1 B) based on alignment with the coding sequence from *Arabidopsis*. This predicted organization was validated by amplifying the full-length sequences from the cDNA of *B. juncea* varieties, Varuna and Heera. The cDNA sequences of homologs 'a' and 'b' from Varuna showed 96.7% sequence similarity with deleted nucleotides at various positions (Supplementary Fig. 1). The protein sequence similarity between the two homologs was approximately 95%, with majority of identical sequences in large segments (Supplementary Fig. 2). Similarly, in Heera, similarity between cDNA and amino acid sequences of the two homologs are 95.07 and 97.4%, respectively (Supplementary Figs. 3, 4).

Identification of polymorphisms between corresponding DEF2 homologs of Varuna and Heera and genetic mapping of the DEF2 gene

Comparative analysis of *DEF2* homolog 'a' sequences revealed variations between Varuna and Heera. ClustalW analysis of homolog 'a' cDNA sequences between the two varieties depicted 99.4% similarity (Supplementary Fig. 5). The amino acid sequences of homolog 'a' from the two varieties showed higher similarity of 99.2% with one amino acid deletion and two substitutions, one of which is a conservative substitution (Supplementary Fig. 6). The effects of these variations between homolog 'a' sequences of Varuna and Heera on protein structure and function was minimal (Supplementary Fig. 7). Homolog 'b' of *DEF2* gene showed 100% similarity in cDNA (and protein) sequences between Varuna and Heera (Supplementary Figs. 8 and 9). Allele-specific primers were therefore designed for homolog 'a' sequences from Varuna and Heera and used for genotyping individuals of the F1DH mapping population and mapping of the gene as described in Materials and methods section. Homolog 'a' was found to co-localize with the A10 linkage group QTL region associated with seed size.

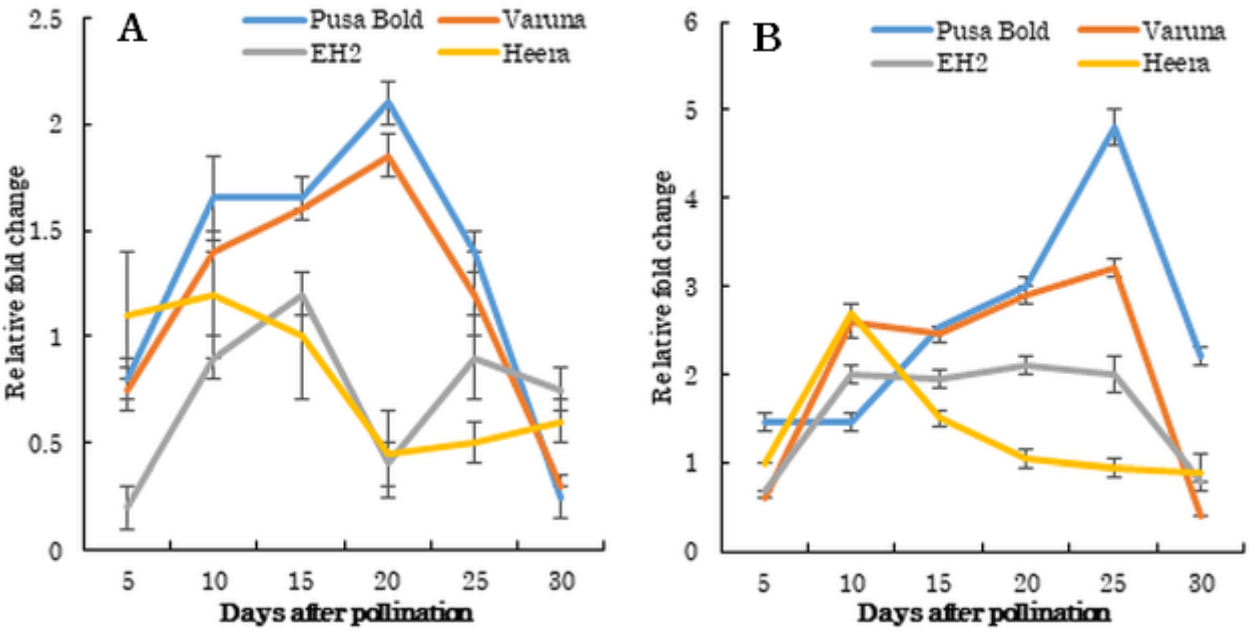

Fig. 2. Expression analysis of *DEF2* homolog 'a' in bold-seeded varieties (Varuna and Pusa Bold) and small-seeded varieties (Heera and EH2) at 5, 10, 15, 20, 25 and 30 days after pollination (DAP) during first growing season (A) and second growing season (B)

Expression analysis of DEF2 homologs during seed developmental stages in bold-seeded and small-seeded germplasm lines

Expression profiles of the two *DEF2* homologs were analysed during various stages of seed development in the bold-seeded Indian germplasm lines (Varuna and Pusa Bold) and two small-seeded East-European lines (EH2 and Heera) in two growing seasons by qRT-PCR (Fig. 2 A, B). Both the bold-seeded Indian varieties showed consistent and significantly enhanced expression levels of homolog 'a' of the *DEF2* gene between ~15 DAP to ~25 DAP as compared to the corresponding stages in small-seeded varieties in both the growing seasons (Fig. 2 A, B). Homolog 'a' exhibited a continuous increase in expression until ~20-25 DAP followed by a significant decline in expression by 30 DAP. Conversely, in the small-seeded varieties, expression of homolog 'a' peaked at 10 DAP and decreased thereafter until 30 DAP. Homolog 'b' did not show any significant variation between the bold- and small-seeded varieties (data not shown). These results indicated that the *DEF2* homolog 'a', which also co-localized with a seed size QTL on A10 linkage group, consistently exhibited higher expression levels throughout seed developmental stages in bold-seeded varieties as compared to small-seeded varieties, indicating a potential correlation between *DEF2* expression and seed size.

Development of transformation vectors and Agrobacterium-mediated genetic transformation of B. juncea vars. EH2 and Varuna

DEF2 homolog 'a', which co-localizes with the major QTL for seed size on A10 linkage

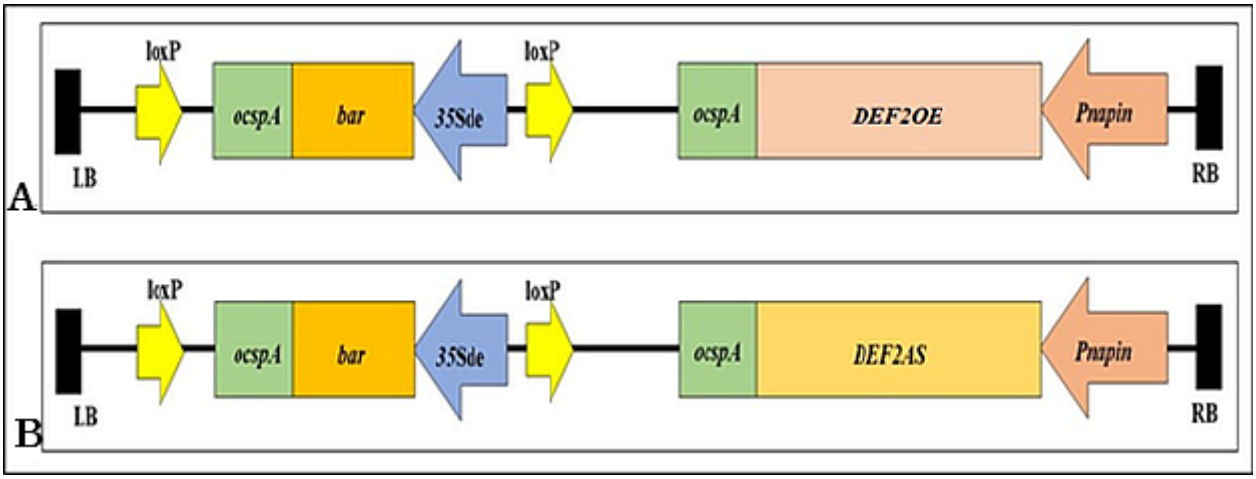

Fig. 3. Schematic representation of the T-DNA region of constructs used for genetic transformation of *B. juncea* var. (A) EH2 and (B) Varuna

group and also showed significant expression level variations between the bold-seeded and small-seeded varieties, was used for genetic transformation experiments to study its functional effects, if any, on seed size. Two *Agrobacterium*-mediated transformation vectors were developed for seed-specific manipulation of *DEF2* expression involving (i) over-expression of the gene in the small-seeded EH2 variety (construct: DEF2OE; Fig. 3 A) and (ii) antisense-mediated down-regulation of the gene in the bold-seeded Varuna variety (construct: DEF2AS; Fig. 3 B). In transformation of EH2 using the DEF2OE construct, we obtained 193 regenerated shoots from 2,515 inoculated explants, indicating a regeneration frequency ranging from 1.4 to 12.1% across seven independent experiments. In Varuna, we obtained 216 regenerated shoots from a total of 900 explants inoculated during co-cultivation, with an average regeneration frequency of ~71.8% in three replicates (Table 1). The transformation frequency for Varuna was significantly higher than that obtained for EH2. We obtained a total of 172 DEF2OE transgenic plants in EH2 and 128 DEF2AS transgenic plants in Varuna. Elongated shoots were transferred to rooting medium and well-rooted plantlets were transferred to a containment net-house in the growing season (Fig. 4 A-D).

Analysis and characterization of transgenic plants

Each T_0 transgenic plant was self-pollinated to obtain T_1 seeds which were

Table 1. Transformation of *B. juncea* using different transformation vectors

Construct	Variety	No. of hypocotyl ends	No. of regenerating hypocotyl ends	Regeneration frequency (%)	No. of shoots transferred to rooting media	No. of transgenic plants
DEF2OE	EH2	2515±42.3	193±4.4	1.4-12.1	193±4.4	172±3.9
DEF2AS	Varuna	900±0.0	647±10.7	67.7-79.0	216±0.0	128±11.8

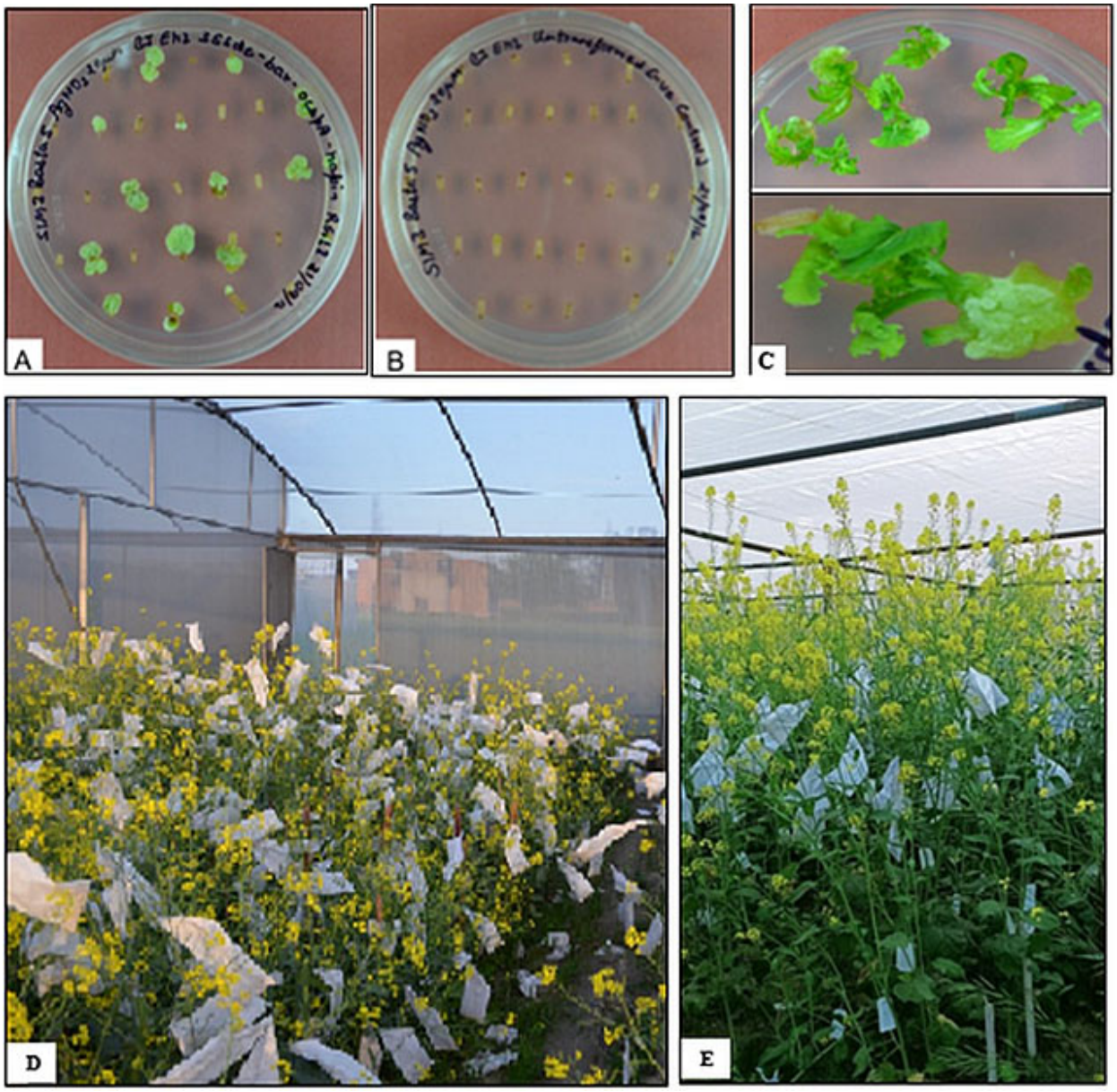

Fig. 4. (A) Transformed shoots on MS media with 2,4-D (0.05 mg l^{-1}), BAP (3 mg l^{-1}), Basta (5 mg l^{-1}), Augmentin (200 mg l^{-1}), glucose (2%) and $AgNO_3$ (20μM) (B) Untransformed (control) explants on above media (C) Well-differentiated shoots transferred to rooting media. (D) Growth and maintenance of transgenic plants in a containment nethouse (E) Maintenance of DEF2OE-29 line.

analysed for their hundred seed weight (HSW) for a preliminary screening of transgene effects on seed size. T_1 seeds from each DEF2OE T_0 plant in EH2 were also germinated and analysed for segregation of Basta resistance/sensitivity. Segregation of Basta-resistant and sensitive plants in a 3:1 ratio indicated single locus integration of the T-DNA. Such events were subjected to molecular analysis by PCR using napin promoter-specific forward primer and *DEF2* gene-

specific reverse primer to confirm the presence of the *DEF2* transgene. Based on the above studies, we identified 17 independent DEF2OE transgenic lines with single-locus integration of T-DNA, of which 11 lines (64.7%) showed prominently higher HSW (ranging between 0.30-0.86 g) compared to the control (untransformed) EH2 plants (0.26±0.01 g). Transgenic plants which had more than one integrated copy of the T-DNA were excluded from further analysis. Similar studies were conducted on T_1 seeds of DEF2AS transgenic lines in Varuna. We obtained 37 single locus DEF2AS transgenic plants of which, 32 plants (~86%) showed reduced HSW in the range of 0.10-0.47 g in comparison to the control (untransformed) Varuna plants (0.49±0.01 g). The DEF2AS seeds were comparatively flattened indicating that downregulation of *DEF2* in Varuna leads to a significant decrease in seed size. These results further validate our observations from DEF2OE transgenic lines in EH2 that the *DEF2* gene contributes positively to seed size and affects seed development. These seeds with reduced size from DEF2AS plants also exhibited poor germination frequencies and were not analysed further.

The single-locus DEF2OE transgenic lines were advanced to T_2 generation by self-pollination of T_1 transgenic plants to study stability of the increased seed size phenotype in subsequent generations and for the development of homozygous lines. Homozygous lines were identified promising transgenic events in the T_2 generation using the approach outlined in the Materials and Methods section. While none of the identified homozygous transgenic plants showed variations in their vegetative and reproductive growth with the untransformed (control) plants, many seeds obtained by self-pollination of the DEF2OE homozygous lines during the 2019-2020 growing season exhibited poor germination frequencies in the subsequent (2020-2021) growing season reasons for which, are discussed in the subsequent section. Our subsequent studies were therefore focussed on one highly promising single-locus homozygous event, DEF2OE-29, which showed consistently good growth and development and did not show any undesirable traits during its entire life cycle across multiple generations, including the seasons wherein other homozygous events faltered due to other issues (Fig. 4 E).

Seed size of open-pollinated seeds of three homozygous DEF2OE-29 transgenic plants were analysed along with untransformed (control) EH2 plants over three growing seasons (2021-22, 2022-23 and 2023-24; Fig. 5 A-C). Growth and seed set of all the transgenic lines was comparable to control EH2 plants. During the 2021-2022 growing season, the TSW (thousand seed weight) of EH2 was 2.8 ± 0.01 g, while DEF2OE-29 was 3.0 ± 0.01 g. In the 2022-2023 season, EH2 was 2.7 ± 0.04 g and DEF2OE-29 was 2.9 ± 0.02 g. For the 2023-2024 season, EH2 was 2.8 ± 0.02 g and DEF2OE-29 remained 3.0 ± 0.01 g (Fig. 5 A-C). Based on Students' t-test, the observed increase in TSW of DEF2OE-29 homozygous lines were significant compared to control EH2 plants in all three growing seasons. We also used other approaches for estimation of seed size. The mean length occupied on graph paper by 50 DEF2OE-29 seeds (9.8 ± 0.03 cm) is significantly larger than that of control EH2 seeds (8.9±0.1 cm) (Fig. 5 D; Table 2). In addition, the number of EH2 seeds per cm^2 was higher as compared to DEF2OE-29 (Fig. 5 E; Table 3). This data indicates that DEF2OE-29 transgenic seeds have a larger seed size compared to

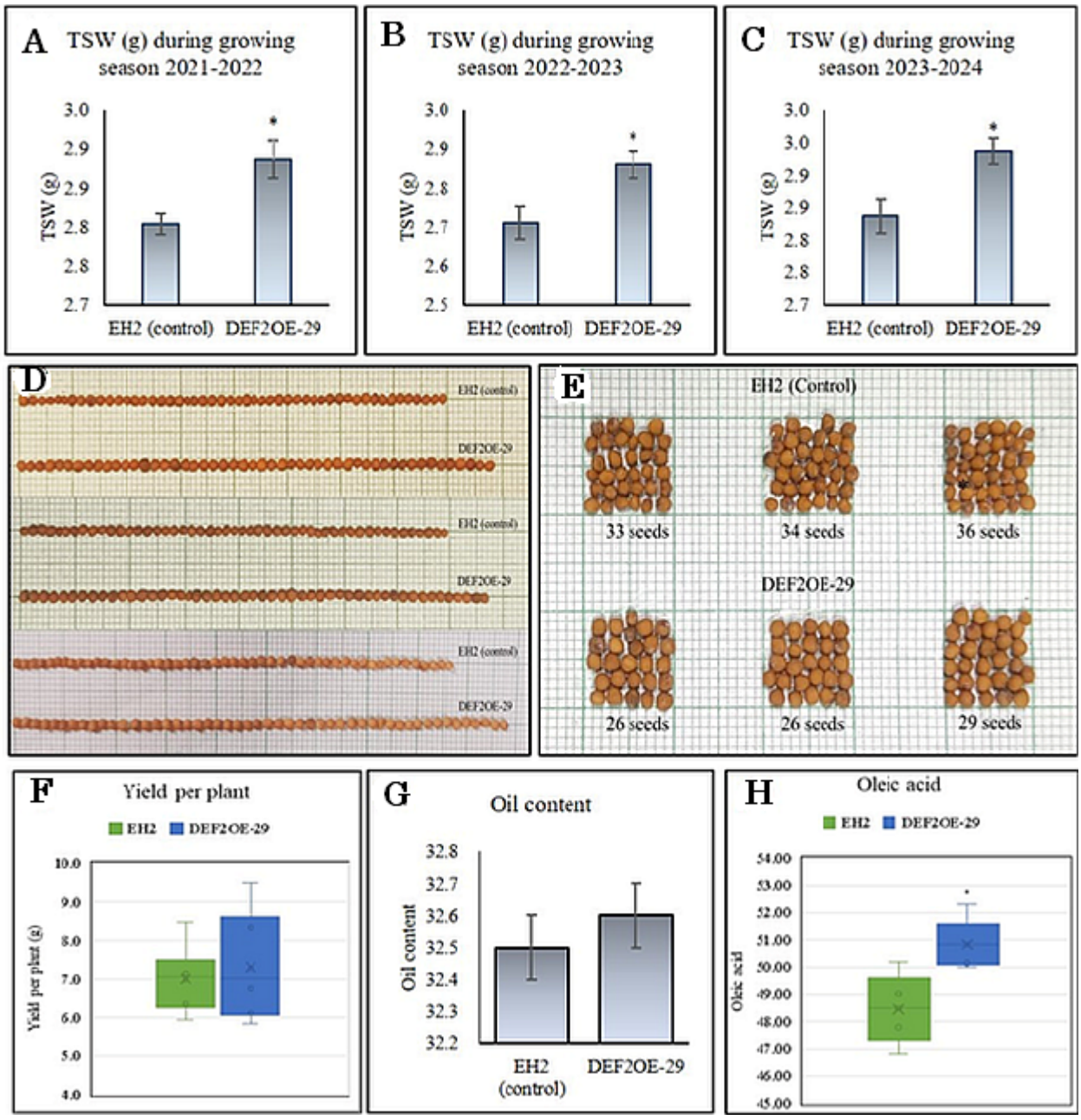

Fig. 5. Mean of thousand seed weight (TSW) (g) of open pollinated seeds of transgenic line DEF2OE-29 and control EH2 plants during three growing seasons (A) 2021-22, (B) 2022-2023, (C) 2023-2024 with n=3; Representation of variations in seed size in terms of (D) length (cm) of fifty seeds, and (E) number of seeds per cm^2 grid area, (F) Yield per plant (n=3), (G) Mean of oil content, (H) Oleic acid content of transgenic line DEF2OE-29 and control EH2 plants (n=5).* indicate significant change at $p<0.05$. n denotes number of replicates

Table 2. Seed size estimation: Length (cm) occupied by fifty seeds from single-locus homozygous DEF2OE-29 transgenics and EH2 (control) plants with three biological replicates. * denotes significant change at $p<0.05$.

Transgenic plants	length occupied (cm) (Mean ± SE)
EBDEF2-29	9.8 ± 0.03*
EH2 (control)	8.9 ± 0.1

Table 3. Number of seeds per cm^2 grid area from single-locus, homozygous DEF2OE-29 transgenics and EH2 (control) plants with three biological replicates. * indicate significant change at $p<0.05$

Transgenic plants	Seeds occupied (cm^2) in grid (Mean ± SE)
EBDEF2-29	27 ± 0.9*
EH2 (control)	34 ± 1.0

control EH2 seeds and corroborates the observed increase in seed size as estimated by average thousand seed weight. Analysis of yield per plant for three homozygous plants were also conducted between homozygous DEF2OE-29 lines and control EH2 indicated no significant change at $p<0.05$ (Fig.5 F). Further, analysis of oil content, fatty acid composition and protein content over two growing seasons in five homozygous DEF2OE-29 transgenic plants using NIRS indicated significant increase in oleic acid content, while no significant change was observed in oil and protein content compared to control EH2 (Fig. 5 G-H).

Discussion

Seed weight and seed size are important agronomic traits in crop plants (Spilde, 1989) and are also one of the key determinants of plant fitness (Larios et al., 2014). Increasing seed weight is often associated with increase in yield (Elliott et al., 2008; Sadras et al., 2008; Bicer et al., 2009). In this study, we report increase in seed size of a small-seeded, East-European variety of *B. juncea* (EH2) by seed-specific over-expression of the *DEF2* gene. Isolation of full length sequences of *DEF2* revealed two distinct fragments, designated 'a" and 'b", indicating the presence of two homologs in both Varuna and Heera. Further analysis of two homologs (a and b) of the *DEF2* gene in Varuna and Heera showed significant sequence similarity in their cDNA (93% in Varuna and 94.5% in Heera) as well as amino acid sequences (95% in Varuna and 97.4% in Heera) indicating conserved functional elements. The inter-specific analysis of homolog 'a' cDNA between Varuna and Heera depicted 98.6% similarity and an even greater similarity of 99.2% in amino acid sequences indicating that the observed variations between corresponding homologs of the two germplasm lines would not lead to significant alteration of protein structure and function. Homolog 'b' of *DEF2* gene showed 100% similarity in cDNA (and protein) sequences from Varuna and Heera. Earlier studies had identified quantitative trait loci (QTL) for seed size using an F1DH mapping population derived from a cross between two *B. juncea* varieties contrasting for seed size: the bold seeded Indian variety, Varuna and small seeded east European variety, Heera (Ramchiary et al., 2007). Mapping of the *DEF2* homolog 'a' in the current study

colocalized the polymorphic gene with a major seed size QTL on A10 linkage group of *B. juncea* in consonance with the predicted co-localization of the *DEF2* gene based on a genome-wide syntenous map between *Arabidopsis* and *B. juncea* (Punjabi et al., 2008). More significantly, expression studies during seed developmental stages indicated prominent variations in homolog 'a' expression profiles between bold-seeded and small-seeded varieties. These observations indicated a strong correlation between *DEF2* gene and seed size phenotype.

Disruption of peptide deformylase activity has been shown to induce albino phenotype in rice and Arabidopsis (Giglione et al., 2000, 2003; Moon et al., 2008). Chloroplast malfunction leads to lower energy, lower assimilation of nutrients and consequent embryo lethality indicating that photosynthetic efficiency is crucial for seed development (Bryant et al., 2011; Li et al., 2021; Zhou et al., 2021; Glowacka et al., 2023). Down-regulation of *DEF2* in the bold-seeded variety, Varuna resulted in seeds that were smaller, flattened and showed poor germination frequencies which lends credence to the above studies. In contrast, transgenic lines based on over-expression of *DEF2* paralog 'a' in EH2 demonstrated significant effects on seed size with 64.7% of single locus DEF2OE transgenic plants showing an increase in seed size of T_1 seeds. For clarity in study of transgene effects, phenotyping of T_1 seeds was done on transgenic plants harboring single-locus integration of the T-DNA. Variation in copy number of T-DNA among independent T_0 transgenic plants is commonly observed due to the production of multiple copies of the T-DNA by *Agrobacterium* and the entry of one or more copies of the T-DNA into the same transformed cell (Dickinson et al., 2023; Gelvin et. al., 2003). Single-locus integration events are preferred in order to facilitate development of homozygous lines for further studies and to ensure genetic stability of the trait. We also observed variations in seed size of T1 seeds from independent single-locus transgenic plants which could be attributed to position effects (De et al., 2022). Moreover, many T0 transgenic plants, by virtue of their growth and maintenance under tissue culture conditions for a long duration, could demonstrate less robust growth as compared to field-grown plants which could also influence seed size. Therefore, in addition to the 11 single-locus transgenic plants that were selected based on preliminary screening of T_1 seeds, it is possible that other transgenic plants, that did not show an increase in seed size of T_1 seeds, could potentially demonstrate more promising phenotypes in subsequent generations.

Many homozygous DEF2OE transgenic lines developed from the identified promising events in 2019-2020 faltered in the subsequent growing season (2020-2021). While the reasons for this observation are not clear, it could have been due to deficiency in plant maintenance, treatment for infections, irrigation during seed-setting stage or other factors as the 2019-2020 growing season, particularly during the flowering and crop maturation stages, coincided with the pandemic-induced lockdown during which, proper execution of these tasks was severely affected. This could have compromised plant maintenance in the net-house leading to production of low-quality seeds/harvest that affected their downstream performance. Subsequent studies on one stable, robust and resilient homozygous *DEF2* over-expression line, DEF2OE-29 in EH2, showed a significant

increase in 1000-seed weight compared to untransformed plants. This increase in seed size was also reflected using other approaches. The observed increase in seed size was however, not accompanied by a significant increase in seed yield per plant or oil content. Similar observations were reported in earlier studies on wheat and soybean, wherein an increase in seed size did not contribute to a corresponding enhancement of seed yield (Milner et al., 2021; Xu et al., 2022).

The global oilseed market is dominated by glycine max and *Brassica napus* (Canola) (Wittkop et al., 2009). *Brassica napus* (rapeseed, canola) is the major source of oilseed production in temperate and subtropical regions (Neik et al., 2020) while *B. juncea* is majorly used for livestock food and biodiesel production (Hossain et al., 2019). In contrast, *B. napus* and *B. carinata* are ranked as sources of oil for biofuel in Canada and in the EU with central Europe being ideal for growing winter varieties of *B. napus* (Ban et al., 2017; Song et al., 2021; Taylor et al., 2010; Wittkop et al., 2009). The ongoing need for improvement and development of oilseed to fit certain niches presents *B. juncea* var. EH2 as a potential candidate. The transgenic lines developed in *B. juncea* var. EH2 could be a promising option for markets in western countries due to its lower erucic acid content compared to the Indian variety, Varuna.

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Author contributions

AJ, BJ and MH conceived and designed the study. BJ and MH conducted all the experiments. AM contributed to development of transgenic plants. BJ, MH and AJ analysed the data. MH, AJ and BJ wrote the manuscript. MA and SG contributed towards data analysis and experimental design. All authors read and approved the manuscript.

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