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Effect of overexpression of *Arabidopsis thaliana SHB1* and *KLUH* genes on seed weight and yield contributing traits in Indian mustard (*Brassica juncea* L. (Czern.))

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Abstract

Seed size is a highly heritable trait in mustard and increasing seed size could improve crop productivity. Here we report the effect of overexpression of two *Arabidopsis* genes *SHB1* and *KLUH* on seed weight and other yield contributing traits in *Brassica juncea*. *KLUH* gene was expressed under its own promoter, whereas the constitutive *HPL* promoter drove *SHB1*. Transgenic status was confirmed through PCR, RT-PCR and Southern analyses. The three *SHB1* events contained single copy of transgene, whereas 12 *KLUH* events carried 1-4 copies of transgenes. All transgenics were fertile and comparable to non-transgenic counterpart for most of the agronomic traits in both T_0 and T_1 generations. Seed size and seed weight in *SHB1* transgenics were 30-40% higher than the control, whereas one *KLUH* event showed significant increase in seed weight. Constitutive overexpression of *SHB1* in *B. juncea* had undesirable pleiotropic effects that were comparable to the *Arabidopsis* lines overexpressing IKU2.

Key words: Indian mustard, seed weight, *SHB1*, *KLUH*, transformation

Introduction

Brassica juncea is an important oil seed crop predominantly grown in the Indian subcontinent. Average yield (1233 kg/ha) of *B. juncea* in India is the lowest among the major rapeseed-mustard producing countries (FAO, 2013). While attempts are being made to develop and deploy hybrids for increasing productivity of mustard, transgenic approaches to improve yield component traits could complement such efforts.

In grain crops, yield is a function of seed weight, size and number. Seed size has been subjected to selection during crop domestication and also by modern plant breeding. Seed size is a quantitative trait and generally there is inverse relationship between seed size and number (Zhang et al. 2011; Yadava et al. 2012). Therefore, improving crop yield through selection for bolder seeds has been a challenge to plant breeders.

Seed size is determined by the coordinated growth of maternal seed coat, zygotic embryo and endosperm tissues. In *Arabidopsis* and many dicot plants, seed development involves an initial phase in which the ovule and endosperm actively proliferate to form a large seed cavity close to its mature seed size. In the second phase, embryo grows to fill the seed cavity at the expense of the endosperm (Sundaresan 2005). Understanding the molecular regulators controlling the growth of these tissues would allow designing strategies to increase seed size. In the past one and a half decades several regulators of seed development and oil accumulation have been deciphered (Li et al. 2008; Zhou et al. 2009; Adamskia et al. 2009). Since seed development in major oilseed crops such as soybean and canola follows very similar path to that of *Arabidopsis*, translating the information gained regarding key regulators of these processes in the model plant could help increase oil yield in crop species (Martínez-Andújar et al. 2012).

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Genes whose overexpression or suppression leads to increase in seed size without drastically affecting seed number are good candidates for engineering seed yield. A number of genes governing seed size such as *DA1*, *ARF2*, *SHB1*, *KLUH* and *AP2* have been identified in *Arabidopsis* (Jofuku et al. 2005;Schruff et al. 2006; Zhou et al. 2009;Adamskia et al. 2009). They are either zygotically or maternally expressed. *SHB1* is a zygotically expressed gene promoting growth of endosperm and embryo during seed development (Zhou etal. 2009). *SHB1* is required for proper expression of two genes involved in endosperm growth, *IKU2* and *MINI3* to promote large seed cavity and endosperm growth, and later for embryo growth through an unknown pathway that is independent of *IKU2* pathway (Zhou et al. 2009). Overexpression of *SHB1* in *Arabidopsis* increased seed mass by 1.5-1.7 fold without affecting seed number (Zhou et al. 2009) suggesting that *SHB1* is a potential candidate for engineering seed size. *KLUH* is a maternally acting gene promoting elongation of integuments of ovules during seed development (Adamskia et al. 2009). Unlike other maternal regulators such as *ARF2*, *DA1* and *AP2*, *KLUH* acts locally in developing flowers to positively affect seed size. While these genes have shown promise for increasing seed mass in *Arabidopsis*, their effectiveness in crop species that have a strong history of selection for the trait remains to be determined. Also, their combined overexpression effect on seed size is not known. In particular, combining *SHB1* and *KLUH* has the potential of coordinated growth of the three seed tissues: endosperm, embryo and integuments required for increasing seed size and consequently seed yield. Therefore, the present study was conducted to explore the possibility of increasing productivity of *B. juncea* through transgenic approach by manipulating seed size through overexpression of *SHB1* and *KLUH* genes.

Materials and methods

Preparation of gene constructs for plant transformation

The*SHB1* gene (*AT4G25350*) wasPCR amplified from *Arabidopsis* using the forward and reverse primers (Table 1). The amplified fragment was digested with *Xba*I and *BamH*I and cloned downstream to the pHPL promoter in the pORE E2 vector (Coutu et al. 2007) (Fig. 1a). The second construct contained the *KLUH* gene (*At1g13710*) driven by its own promoter. First, a 3.7 kbp upstream region of *KLUH* gene was PCR amplified using *Arabidopsis* genomic DNA as template.

Table 1. List of primers used in the study

Name	Sequence (5'-3')	nt
KLUH-F	gatCCCGGGATGTCTCCGGAAGCT	24
KLUH-R	atGGTACCTCAAGCGAAACCAACA TTCCTTG	31
pKLU-F	cgtGCTAGCGTTCCCAAAAGTCAT AAATGTAGTTC	35
pKLU-R	taCCCGGGTTTATGAAGAAGATATG AAGTGTGTGTG	36
SHB-F	acgGGATCCATGAGGTTTGGGAAA GAGTTTG	31
SHB-R	ataCTGCAGCTAATTGTTATGATGA TCTCCATC	30
qSHB1-F	ACGGAACTTACTGGGACATTG	21
qKLUH-F	GACATGATTGCTGTTCTTTGGG	22
Tnos-R1	CTTTATTGCCAAATGTTTGAACGATC	26
NPT II-F	CAAGATGGATTGCACGCAGG	20
NPT II-R	GAATCGGGAGCGGCGATA	18
GAPDH-F	TCAGTTGTTGACCTCACGGTT	21
GAPDH-R	CTGTCACCAACGAAGTCAGT	20

The amplified fragment was digested with *Nhe*I and *Xma*I and cloned into pORE O4 vector. Next, the *KLUH* gene was PCR amplified using the forward and reverse primers (Table 1). The amplified fragment was digested with *Xma*I and *Kpn*I restriction enzymes and cloned downstream of *KLUH* upstream region (Fig. 1b). Finally,*SHB1*and *KLUH* gene constructs were transformed into *Agrobacterium tumefaciens* strain GV3101.

Transformation of B. juncea

Hypocotyl explants (0.5-1.0 cm) of *B. juncea* cv. RLM 198 from the 6-day-old seedlings were cultured for two days on BD medium (MS medium containing 1 mg/ml BAP and 0.1 mg/ml 2,4-D, 20 mM $AgNO_3$ and 0.8% agar). *Agrobacterium* culture was grown in yeast extract peptone medium containing 20 mg/l rifampicin, 25 mg/l gentamycin and 50 mg/l kanamycin at 28°C to reach OD_{600} of 0.5-0.6. Pre-cultured explants were incubated in 20 ml bacterial suspension (OD_{600} 0.1-0.2) for 20-30 min. After infection, explants were blot dried and placed on fresh BD medium, and kept in dark at 25±2°C for 2 days. Following co-cultivation, explants were washed twice with liquid BD medium containing 500 mg/l cefotaxime. After three days, explants were cultured on BD medium supplemented with 500 mg/l cefotaxime and 50 mg/l kanamycin till green shootsd differentiated from explants. Green shoots were transferred to the shoot elongation medium (MS medium with 0.2 mg/lBAP, 50 mg/l kanamycin

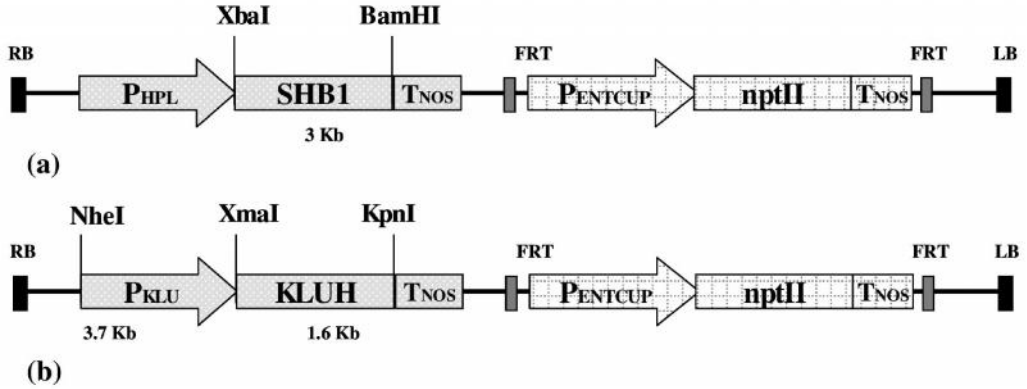

Fig. 1. Plant transformation vector constructs. (a) *KLUH* gene was cloned downstream of its 3.7 kbp upstream region into pORE-O4 binary vector using *Nhe*I, *Xma*I and *Kpn*I restriction sites and (b) *SHB1* gene was cloned downstream of the *HPL* gene promoter in pORE-E2 binary vector using *Xba*I and *Bam*HI restriction sites

and 500 mg/l cefotaxime). Elongated shoots were transferred to MS medium with 2 mg/l IBA, 20 mg/l kanamycin and 500 mg/l cefotaxime for rooting. Plantlets were hardened and transferred to pot containing mixture of soil, compost, peat, and gypsum.

Molecular analysis of transgenic plants

PCR analysis of T_0 plants was carried out for the *nptII* marker gene and thetransgenes using forward and reverse primers (Table 1). *nptII* positive plants were further screened with four pairs of vector backbone primers to test for*Agrobacterium* contamination in the plant DNA samples (Table 1). Transgene expression was tested by RT-PCR using RNA extracted from leaves and developing ovules (15-22 days-after-pollination, DAP). Copy number of T-DNA insertions in transgenic plants was determined by Southern analysis. Genomic DNA fragments obtained by digestion with *EcoR*V were separated by electrophoresis on a 0.8% agarose gel, blotted onto nylon membrane (Hybond N^+, Amersham) and immobilized by UV cross linking. Hybridization was carried out with 0.7 kb *nptII* gene fragment as probe labeled with α^{32}P-dCTP. Hybridization was conducted overnight at 60°C, followed by three stringent washes with 2X, 1X and 0.5X of 10% SDS. After washing, the membrane was exposed to X-ray film for a week at -80°C to obtain autoradiogram.

qRT-PCR was performed using SYBR FAST qRT-PCR Master mix (2x) (KAPA Biosystems) to determine the levels of *KLUH* and *SHB1* transcripts in the respective T_1 transgenic plants. Total cDNA was diluted to 25 ng/µl and 100 ng was used in a 10 µl reaction mixture. For each reaction, three technical replicates, no template control (NTC) and no primer control were used to check for the contaminants. The following programme was used for qRT-PCR reactions: 3 min at 95°C, 3 sec at 95°C and 30 sec at 60°C for 40 cycles, which includes data acquisition. Finally, a dissociation curve analysis was performed from 65°C to 95°C in increments of 0.5°C, each lasting for 5 sec. Amplification of SYBR green-labeled PCR fragments was achieved by using gene-specific forward and T_{NOS} reverse primers (Table 1). *GAPDH* was used as the control in qRT-PCR. Expression value of *KLUH* in ovules of KLUH1 event was taken as one to determine the relative expression level (expressed as $\log_2$ fold difference) in other samples.

Analyses of growth and yield parameters of T_1 transgenic plants

Two SHB1 events, four KLUH events and control plants were evaluated for phenotypic performance in a completely randomized design. Observations on growth and yield contributing traits such as plant height, number of primary branches, number of secondary branches, days-to-flowering, period of flowering, number of siliques per plant, silique length and number of seeds per silique were recorded on three plants for each plant. Data were statistically analyzed to find significant differences between transgenics and wild type plants. Oil content was determined using the MQC benchtop NMR analyzer (Oxford Instruments).

Results

PCR for *KLUH* gene, *KLUH* upstream region and *SHB1* gene amplified 1.6 kbp, 3.7 kbp and 3 kbp amplicons, respectively. *KLUH* gene was cloned under its own promoter in the pORE-O4 binary vector and *SHB1* gene was cloned in the pORE-E2 binary vector under the *A. thaliana HPL* gene promoter (Fig. 1). Cloning was confirmed by restriction digestion and sequencing.

Restriction digestion showed the expected size of inserts (Fig. 2) and fidelity of cloned fragments was confirmed by sequencing. Subsequently, recombinant vectors were mobilized into *Agrobacterium* strain GV3101.

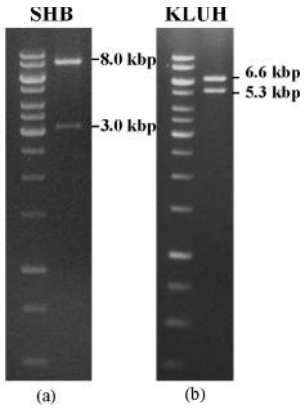

Fig. 2. Restriction digestion of transformation vector constructs. a) Gel picture of pORE-O4 plasmid digested with *Nhe*I and *Kpn*I restriction enzymes showing 5.3 kbp *KLUH* gene/promoter and 6.6 kbp vector sequence, b) Gel picture of pORE-E2 plasmid digested with *Xba*I and *Bam*HI restriction enzymes showing 3.0 kbp *SHB1* coding sequence and 8.0 kbp vector sequence

Co-cultivated explants gave rise to small calli at the cut ends, followed by differentiation of shoot buds after 3-4 weeks of culture on shoot induction medium. In transformation experiments, 87 of the 1400 explants infected with *Agrobacterium* carrying *KLUH* gene construct gavegreen shoots (transformation frequency of 6.5%). Likewise, 20 of the 500 hypocotyl explants infected with *Agrobacterium* containing *SHB1* construct produced green shoots at overall transformation frequency of 4%. Green shoots obtained after four weeks of culture on SEM readily rooted in IBA-containing medium. Well-rooted plantlets were hardened and transferred to pots. Finally three events of *SHB1* and 12 events of *KLUH* gene constructs were generated.

Molecular analyses confirmed thepresenceof transgene in all transgenics

PCR screening of T_0 transgenic plants showed presence of both *nptII* (Fig. 3a) and transgene. To rule out false positive PCR due to *Agrobacterium* contamination, transgenics were further screened with four pairs of vector backbone specific primers. Of these, one primer pair each targeted the region just adjacent to the left and the right borders of T-DNA. None of the transgenics gave amplicons of backbone fragments confirming that nptII and transgene amplicons came from T-DNA inserted in the host genome. Further, absence of backbone sequences indicated that T-DNA insertions in these events are clean and devoid of aberrations.

Southern hybridization revealed single copy of transgene in all three *SHB1* transgenic events whereas in *KLUH* transgenics copy number varied from one to four. Three out of 12 KLUH transgenic events had single copy insertion whereas others had two or more copies (Fig. 3b). In each of the transgenics the shortest fragment visualized was longer than the T-DNA fragment (~2.18 kbp region, see Fig. 3b) corresponding to the probe indicating lack of truncations in integrated copies.

Multicopy integrations may be linked or unlinked. Linked transgene copies may integrate in head-to-tail, tail-to-tail or head-to-head orientation. With the restriction-probe combination tested here, tail-to-tail integration of transgenes would yield 4.36 kbp Southern band. The fragment size analyses of blots showed none of the transgenic events had 4.36 kbp fragment indicating absence of multicopy tail-to-tail insertions. Head-to-tail insertions would show a 8.09 kbp *EcoR*V fragment in *KLUH* transgenics and 7.06 kbp fragment in *SHB1* transgenics. A fragment of 8.03 kbp size was detected in Southern blot of KLUH6 event. However, such a fragment could also result from integration of normal T-DNA copy. Therefore, all transgenics appear to carry clean T-DNA fragments.

qRT-PCR shows variation in SHB1 and KLUH expression among transgenic lines

The transgenics were analyzed by RT-PCR for expression of transgene using *GAPDH* gene as the control. We designed the reverse primer from the NOS terminator, and forward primer from the transgene region to amplify transgene specific transcripts (Table 1). RT-PCR showed expression of transgenes in developing ovules of all the transgenics (Fig. 3c). Further, qRT-PCR was carried out to quantify the expression of transgenes in different tissues. Expression of *KLUH* transgene was the highest (32 fold) in leaf followed by flower buds (5.6 fold) and ovule (1.3 fold). However, expression level of *SHB1* transgene was higher in flower buds followed by leaf and ovules (Fig. 3d). Further, we tested the expression levels of *SHB1* gene in SHB1-1 and SHB1-2 events. Expression level of *SHB1* gene in leaf tissue was 2.5 fold higher in SHB1-2 event compared to SHB1-1 event (Fig. 3e). This variation in transgene expression levels in the different events of SHB1 construct could be

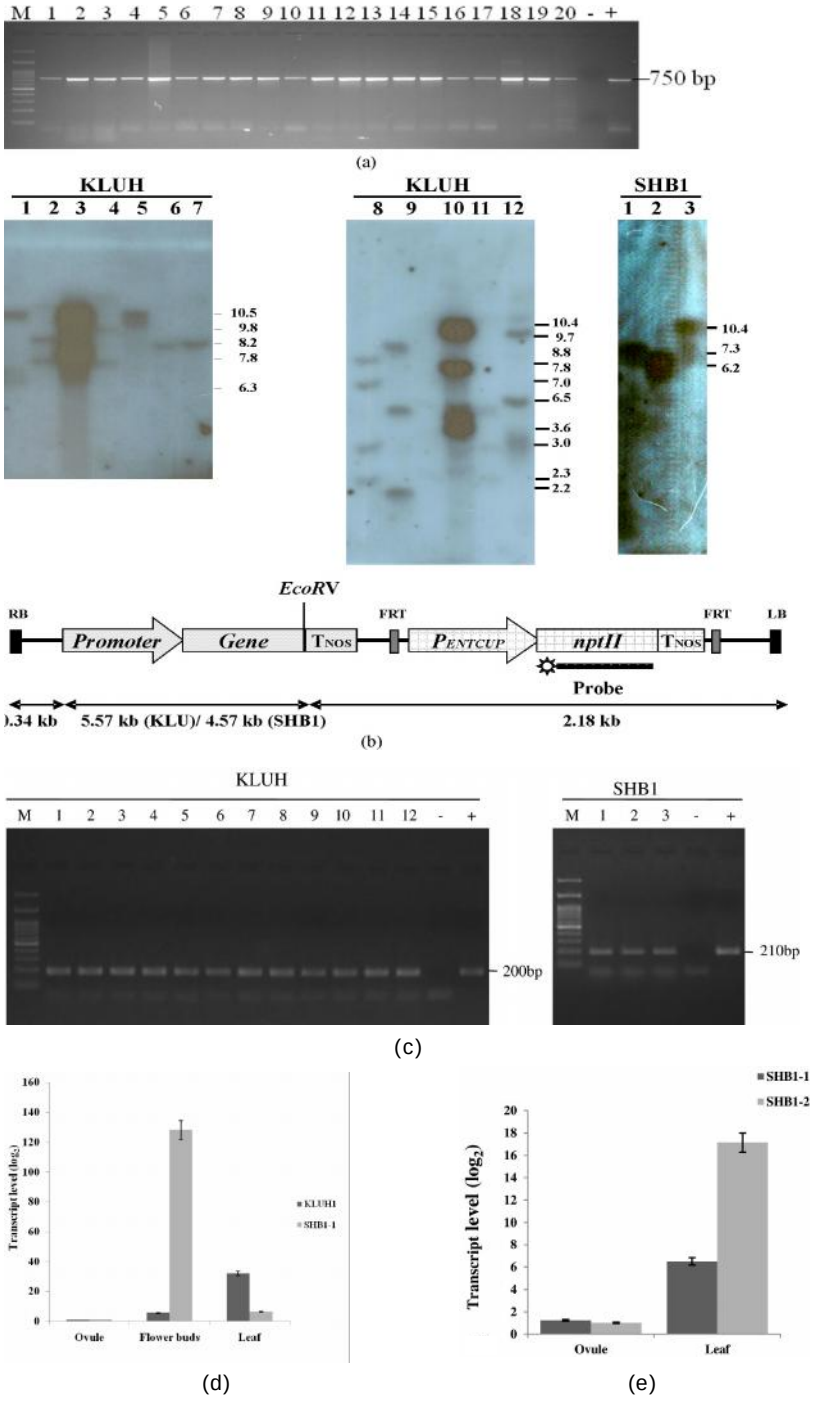

Fig. 3. Molecular analyses of *B. juncea* transgenics. (a) Confirmation of transgenic status by PCR with *nptII* primers, (b) Southern blots of transgenics prepared by digesting total DNA with *Eco*RV and probed with *nptII* gene, (c) RT-PCR amplification of KLUH and SHB1 genes using gene specific forward primer and NOS terminator reverse primer (fragment sizes in kbp are shown on the right), (d) qRT-PCR of *KLUH* and *SHB1* genes in different tissues and (e) qRT-PCR showing expression levels of *SHB1* gene in ovule and leaf tissues of SHB1-1 and SHB1-2 transgenic events. M = molecular weight marker, - = negative control (untransformed plant DNA), + = positive control (plasmid DNA)

due to position effect.

SHB1 overexpression increases seed weight

All T_0 transgenic events except the SHB1-2, grew, flowered and set seeds like non-transformed plants. However, owing to constraints of space and timely availability of seeds, two out of the three *SHB1* events and four out of 12 *KLUH* transgenics were carried forward to T_1 generation and analyzed for growth and yield traits. As T_1 seeds were sown directly into pots without using kanamycin selection, 10 randomly chosen plants of each of the T_1 events were PCR screened using *nptII* primers. Data on morphological traits were recorded on three *nptII* positive plants picked at random for each event.

No significant difference was recorded among transgenics and control for plant height, number and length of silique, and number of secondary branches. Likewise, except for SHB1-2 event, transgenics did not differ from the control for days-to-flowering. There were large variations among transgenics for traits such as number of primary branches, seeds per silique, seed weight and seed size (Fig. 4a-c). The most prominent difference was recorded for seed weight. *SHB1* transgenics produced significantly heavier and larger seeds than untransformed control plants. SHB1-1 and SHB1-2 plants recorded mean 100-seed weight of 405 mg and 435 mg, respectively as compared with 311 mg in the control plants. Thus, on average *SHB1* plants bore 35% heavier seeds than the control. However, SHB1-2 event displayed other morphological changes. Leaves were crinkled, flowering was delayed by a week and the flowering period was extended by 10 days. Further, flower buds and flowers of SHB1-2 plants were larger and pistils were elongated (Fig. 5). As a consequence, at anthesis, stamen

were placed well below the stigma, which affected seed set. Siliques of SHB1-2 plants were broad and seeds were flat rather than spherical (Fig. 5). 100-seed weight of KLUH transgenics ranged from 319-380 mg. Only KLUH3 event showed significantly heavier seeds than the control. Transgenics differed for seeds per silique and KLUH1 and SHB1-2 events registered significantly fewer seeds per silique than the control. In general, seed oil content was low in all treatments including the control perhaps due to inadequate growth conditions in the Phytotron. Oil content could not be determined in KLUH1 and SHB1-2 events due to insufficient quantity of seeds produced per plant. In the rest, seed oil content ranged from 20-23% (Fig. 4d).

Discussion

Among the yield contributing traits in oil seed Brassicas, seed weight and seeds per silique show high heritability (Yadava et al. 2012; Zhang et al. 2011). Although several QTLs governing seed size/weight have been reported (Fan et al. 2010; Yang et al. 2012), underlying genes have not been identified so far. Further, seed size is negatively correlated with seeds per silique and some of the QTLs for the two traits show colocalization (Chen et al. 2011;Yadava et al. 2012). Therefore, increasing seed weight without compromising seeds per silique has been a challenge. In this study, we attempted to manipulate seed weight of mustard through transgenic approach by over expression of *KLUH* and *SHB1* genes of *A. thaliana*. We generated over 100 green shoots through *Agrobacterium*-mediated transformation. We used 50 mg/l kanamycin for selection of transgenic plants and our transformation frequencies were lower than reported earlier (Singh et al. 2009), but we obtained escape-free transgenics.

Transgenic status of plants was conclusively established through PCR, Southern and RT-PCR. In particular, our strategy of checking for T-DNA specific and plasmid vector backbone specific regions by PCR was very effective in determining true transformants at the early stage of shoot regeneration. *Agrobacterium*-mediated transformation often gives rise to plants carrying vector backbone sequences in addition to T-DNA. In our case, none of the transgenics carried backbone sequences. Although plants with 1-4 copies of T-DNA were obtained, nearly half of the transgenics had 1-2 copies of T-DNA. Further, Southern analysis indicated that there was no

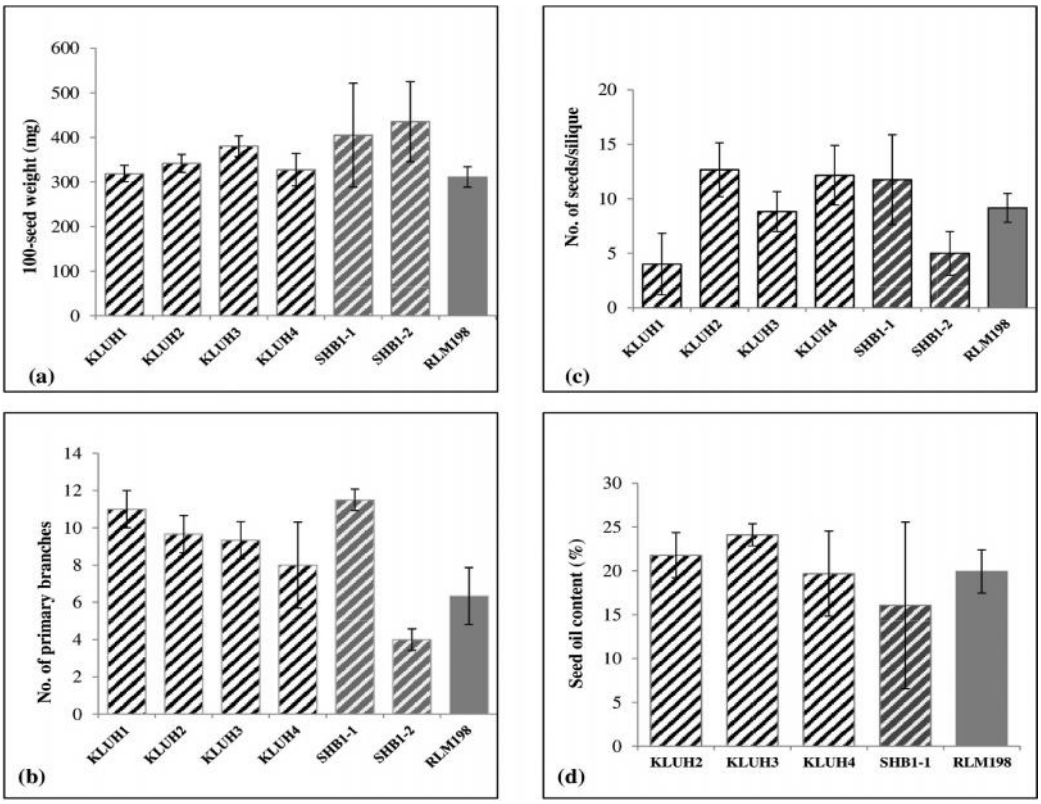

Fig. 4a-d. Comparative performance of selected KLUH and SHB1 T_1 transgenic events and control (RLM 198) for (a)100-seed weight (b) number of primary branches, (c) number of seeds per silique and (d)seed oil content

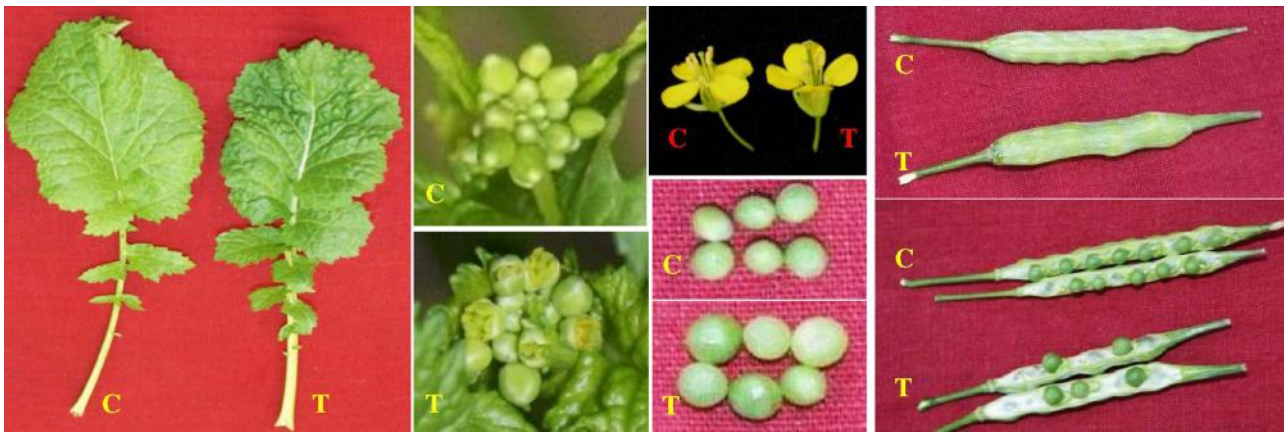

Fig. 5. Phenotype of SHB1-2 (T) and the control RLM 198 (C). Transgenic plants have crinkled leaves, bigger and slightly opened flower buds, longer pistils but normal size anthers, larger seeds, wider siliques with fewer seeds

rearrangement of T-DNA during transformation. RT-PCR results showed that the transgenes are expressed in each of the transformants. These results suggest that the protocol used is optimal and yields clean transgenics.

All transgenics were male and female fertile, and showed nearly normal phenotype for most of the agronomic traits except seed weight. Both events of *SHB1* showed significantly larger and heavier seeds than the control and indicated that overexpression of this gene is effective in increasing seed weight. On the other hand, overexpression *KLUH* gene did not show consistent increase in seed weight. *SHB1* expression in *Arabidopsis* is restricted to embryo/ endosperm (Zhou et al. 2009). In the present study, we used a constitutive promoter pHPL to drive the *SHB1* gene. The*SHB1* expression pattern found in transgenic mustard plants was comparable to the expression of *HPL* gene in *Arabidopsis* (Winter et al. 2007). This constitutive expression might have led to pleiotropic effects in *SHB1* transgenics. *SHB1* regulates expression of *MINI3* and *IKU2* genes in *Arabidopsis* (Zhou et al. 2009). While *IKU2* overexpression has been shown to yield larger seeds in *Arabidopsis*, *MINI3* overexpression did not have any effect (Fatihi et al. 2013). Further, use of soybean glycinin promoter to overexpress *IKU2* gene showed maternal effect on seed size and the silique were thicker. This was attributed to ectopic expression of *IKU2* in maternal tissues by the glycinin promoter (Fatihi et al. 2013). In our studies too, maternal effect of *SHB1* overexpression was evident as T_0 transgenics showed larger seeds. Considering that *SHB1* exerts its effect via *IKU2*, our results corroborate the above results. In SHB1-2 event pistil was elongated while stamens were of normal size. This affected pollination that led to poor seed set. Similar anther-pistil mismatch was found in *Arabidopsis* when *BnGRF2* gene was constitutively expressed using 35S promoter (Liu et al. 2012). Therefore, to avoid adverse pleiotropic effects, *SHB1* expression needs to be restricted to embryo and endosperm.

Seeds of *IKU2* overexpression lines of *Arabidopsis* were enriched with fatty acids but total seed yield per plant was less than the control (Fatihi et al. 2013). In our case, oil content of transgenics and control plants were lower compared to previous studies in mustard (Gangapur et al. 2009; Singh et al. 2011). This is attributed to inadequate environmental quality under containment glass house conditions. Nevertheless, seed yield and seed oil content of *KLUH* and *SHB1* transgenics were higher than control plants.

At present, majority of researches on improving oil yield in crops like rapeseed, soybean through transgenic technology have focused on genes of the fatty acid biosynthesis pathway (Vigeolas et al. 2007; Rao et al. 2009). However, there are no reports on exploiting key genes identified to be involved in seed development in *Arabidopsis* for improving seed size and seed yield. Our study shows that expression of *SHB1*, and perhaps *KLUH*, the two key genes involved in seed development could improve seed weight. Further studies need to be conducted under field conditions to validate the results.

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