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Pyramiding and evaluation of segregating lines containing lectin and protease inhibitor genes for aphid resistance in *Brassica juncea*

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Aphids are one of the most devastating pests, affecting the potential yield and quality of*Brassica juncea*. In the current study, we have attempted to pyramid two transgenic lines containing chickpea lectin (CHPL, P_1) and urdbean protease inhibitor (UPI, P_2) in each under the phloem specific rolC promoter, through conventional breeding approach. In the derived F_2 population, both lectin and protease inhibitor genes were segregating in a 9:3:3:1 ratio (p-value: 0.81), indicative of a single copy of the transgenes in the parents. Furthermore, the parental, as well as pyramided progenies were evaluated for their potential resistance to aphids in terms of mortality and natality. The lines containing both the transgenes were found to be superior over single gene transgenics as a higher mortality rate (96%) was found in F_2on the 9th day as compared to single gene transgenics (86% and 80% in P_1 and P_2 respectively). A significant decrease in the number of nymphs was observed in P_1 and P_2 but most in F_2 plants as almost 43, 32.08, and 107.5 times decrease in the number of nymphs was found in P_1, P_2, and F_2 individuals over control. Expression profiling was done to see if there was any impact of gene pyramiding on the expression pattern of both transgenes before and after aphid treatment, and no significant changes were observed, indicating constitutive expression of transgenes in pyramided lines also. In conclusion, pyramided lines were found to be promising and were superior for aphid resistance.

Keywords: Transgenics, Transgenic breeding, Chickpea lectin, Urdbean protease inhibitor

Indian mustard (*Brassica juncea* L. Czern) is one of the most important oilseed crops[1], consumed worldwide due to its high unsaturated fatty acid content and other supplements[2]. Despite being the 4th largest edible oil economy after the United States, China, and Brazil, India is the second largest importer of edible oil after China, spending over 0.6 trillion dollars annually to supplement domestic supplies (3rd National Brassica Conference, New Delhi, 2017). Being an annual herb, Indian mustard encounters several biotic and abiotic stresses throughout its life. Insect pests are the most common among the biotic stresses, causing serious harvest losses[3].

Sap sucking insects like *Lipaphis erysimi* and *Myzus persicae* are the most devastating insects, infesting leaves, stems, and floral parts, causing 35.4-91.3% yield loss[4,5]. Multiple pesticide sprays are required in order to minimize the damage caused by pests[6], which aphids might escape being sticked to the abaxial surface of the leaves. Systemic insecticides are absorbed and circulated through phloem[7], hence they are more effective but at the same time associated with dangerous implications on the environment and non-target organisms[8].

The best way to tackle losses due to aphid infestation is to identify and adopt aphid resistant cultivars. The mining of resistant genes against aphids from natural germplasm has been explored, but due to lack of resistance in the cross compatible gene pool, varietal improvement against it through conventional breeding is difficult. Attempts have been made to screen wild crucifers for resistance to *L. erysimi*. After rigorous screening, *B. fruticulosa* was found to be resistant as all the released aphids died within 5-8 days[9]. In another experiment, *B. montana* was found to possess high resistance against aphids[10],

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Suppl. Data available on respective page of NOPR

which can be a potential donor line but is associated with linkage drag and cross incompatibility with the cultivated gene pool. Hence, *B. fruticulosa* and *B. montana* are not suitable for gene introgression through conventional breeding, therefore, genetic engineering mediated transfer of resistance genes could be a potential approach.

Use of insecticidal transgenes such as lectin and protease inhibitor (PI) genes were recommended as an alternative to pesticide spray[11,12]. The first plant lectin found to have insecticidal activity towards aphid species was *Galanthus nivalis agglutinin* (GNA), which showed improved resistance against the green peach aphid (*M. persicae*) when transformed into tobacco[13], Transgenic tobacco and Indian mustard plants expressing *Allium sativum* agglutinins lectin (ASAL) exhibited partial resistance to aphids with reduced survival and fecundity[14]. Transgenic *B. juncea* expressing GNA, *Allium cepa* agglutinin (ACA), and an ASAL: ACA fusion protein negatively affected the population of *L. erysimi*[15]. The wheat germ agglutinin (WGA) expressed in *B. juncea* shows anti-metabolic, anti-feedant activities against *L. erysimi*[16]. Further *Vigna radiata* and *Cajanus cajan* derived lectins have been overexpressed in *B. Juncea* for the development of resistance against aphids[17,18]and promising results were obtained.

Protease inhibitors are capable of regulating several endogenous functions like acting proteases, modulation of programmed cell death, stabilization of defense proteins, targeting the digestive proteases of pathogen[19]. Also, hemipteran insects use cysteine protease as the primary digestive protease[19] transforming *B. juncea* with a cysteine protease inhibitor could be a promising approach for aphid control. The PI gene from *V. mungo* was found to be promising against *L. erysimi* when overexpressed in mustard[20]. Since, the cysteine PI oryzacystatin I (OC-I) was found to be effective against various aphids after an artificial diet bioassay[21,22], transgenic oilseed rape expressing the OC470 I gene was developed, resulting in reduced aphid infestation[21]. Recently, some researchers have characterized few important traits in brassica along with other crops and microbes by application of genetic and metabolic engineering technology to enhance the crop yield and quality[23-25].

Co-expression of two or more resistance genes into the same plant, particularly those with completely different modes of action, could be a useful strategy for achieving higher insecticidal activity, broader protective spectra, and increased durability of transgenic plants[26,27]. Keeping these in mind, in the current study we have attempted the introgression of the chickpea lectin (CHPL) and urdbean protease inhibitor (UPI) genes present as a transgene in the Varuna variety of brassica through conventional breeding followed by assessment of inheritance pattern, expression analysis, and aphid bioassay for mortality and natality in parental lines as well as a segregating population. The current study will pave the way for resistance breeding for aphid resistance in brassica.

Materials and Methods

Plant material and isolation of genomic DNA

Transgenic *B. juncea* lines developed for aphid resistance having a chickpea lectin gene (HM235917.1) (P_1) and an urdbean protease inhibitor (HQ629949) (P_2) were used as parent material for the whole experiment. These lines were advanced from T_0 to T_3 generation to fix the transformation event by continuous selfing in the polyhouse at ICAR-NIPB, New Delhi. The T_3 generation plants were taken as parents to pyramid both transformation events through a conventional breeding approach. Before crossing, both P_1 and P_2 plants in the T_3 generation were further screened using the transgene specific primers.

The F_1 plants obtained after crossing P_1 and P_2 plants were advanced to the F_2generation, comprising 471 plants through selfing. Leaf samples of P_1, P_2, F_1, F_2, and control (Varuna, non-transgenic) plants were collected and used for DNA isolation using the modified CTAB method[28]. The quality of genomic DNA was analyzed on a 0.8% agarose gel using Lambda DNA (Fermentas, USA) as a reference and quantified using Nanodrop (ND-1000) (Thermo Scientific, USA).

Screening and selection of F_1 hybrids and F_2populations using transgene specific primers

The PCR reaction was programmed with one step denaturation at 93C for 4 min, subsequent 35 cycles of 93°C for 1 min, annealing of the primer at an optimum temperature (Table 1) for the 30s, and extension at 72°C for 45 s. The final chain extension was done at 72°C for 7 min. The amplified PCR products were separated on a 1.5% agarose gel and the amplicon size was estimated using a 1 kb and 100 bp gene ladder (O' Gene-Ruler) as a reference.

Table 1 — Primers used for Screening of Transgenic brassica plants and gene expression analysis

S. No.	Transgene	Primers	Sequences	Tm (°C)	Product size (bp)
		Gene specific primers			
1	Chickpea lectin	Forward	ATGGCTTCTCTT CAAACCCA	56.5	825
		Reverse	TGCATCTGCAG CTTGCT		
2	Urdbean protease inhibitor	Forward	ATGATGGTGCT AAAGGTGTGTG	56	310
		Reverse	CCATGGATTTGC AAGGTTTGT		
		Primers for expression analysis			
3	Chickpea lectin	Forward	TGAACGGGCTA ATGTTGTGA	55.6	NA
		Reverse	ACCAATCCTCA CCCACTCAG		
4	Urdbean protease inhibitor	Forward	GCCGCTGCACT AAATCAATAC	57.9	NA
		Reverse	TGTCAAGGCAA CGACACTT		
5	Actin (AT3G18780.2)	Forward	TGTGCCAATCTA CGAGGGTT	58.5	NA
		Reverse	ACAACGGCACT ACTGGATCA		

RNA isolation and cDNA synthesis

Due to the fact that both transgenic constructs in P1 and P2 were derived from the phloem-specific rolC promoter triggered by sucrose[29], the transgenes were expressed constitutively in phloem cells. The fresh leaf samples were taken from P_1, P_2 as well as F_2 plants before, and on the 5th and 9th days after aphid infestation and kept in liquid nitrogen. Total RNA was isolated using Spectrum Plant Total RNA Kit (SIGMA), the RNA isolated from F_2 plants was pooled at an equal concentration. RNase-free DNase (Ambion) treatment was given to 10 µg of total RNA. Isolated RNA was quantified by Nanodrop (ND-1000) and RNA integrity with quality was checked by 1.2% denaturing agarose gel electrophoresis. The RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA), was used for cDNAsynthesis as per the manufacturer's protocol.

Expression analysis of the transgene

Due to the fact that both transgenic constructs in P1 and P2 were derived from the phloem-specific rolC promoter triggered by sucrose[29], the transgenes were expressed constitutively in phloem cells. The fresh leaf samples were taken from P_1, P_2 as well as F_2 plants before, and on the 5th and 9th days after aphid infestation and kept in liquid nitrogen. Total RNA was isolated using Spectrum Plant Total RNA Kit (SIGMA), the RNA isolated from F_2 plants was pooled at an equal concentration. RNase-free DNase (Ambion) treatment was given to 10 µg of total RNA. Isolated RNA was quantified by Nanodrop (ND-1000) and RNA integrity with quality was checked by 1.2% denaturing agarose gel electrophoresis. The Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, USA), was used for cDNA synthesis as per the manufacturer's protocol.

Aphid bioassay

After PCR amplification with a transgene specific primer, 10 plants from both P_1 and P_2 parents; and 10 from the F_2 population (containing both transgenes), were used for the aphid bioassay. Twenty adults of *L. erysimi* were allowed to feed on transgenic as well as control plants. The percent mortality of aphids was calculated as suggested by[30], and the formula is provided below.

$$\text{No. of dead insects} = \frac{\text{No. of insects allowed to feed}}{\text{No. of insects eloped}} \times 100$$

The phenotypic data such as plant height (cm), length of silique, seeds/silique, 1000 seed weight (g), and seed yield/plant (g) of non-transgenic (control) and transgenic (P_1, P_2, and selected F_2) plants were also observed (Table 3).

Analysis of agronomic traits

Transgenic *B. juncea* (T_3) having CHPL and UPI genes was screened for the presence of the transgene by gene-specific primers (Fig. 1A & B), Crossing of positive P_1 and P_2 plants was done and true hybrid F_1 plants were selfed to generate F_2 plants (Fig. S1A & B). After screening of the resultant 471 F_2 plants by gene-specific primers for both transgenes, 355 plants were found to be positive for chickpea lectin, while 351 plants were found to possess an urdbean protease inhibitor in them. A pictorial representation for both is provided (Fig. 2A & B).

Results and Discussion

Selection of F_2 introgression lines with both transgenes

Transgenic *B. juncea* (T_3) having CHPL and UPI genes was screened for the presence of the transgene by gene-specific primers (Fig. 1A & B), Crossing of positive P_1 and P_2 plants was done and true hybrid F_1 plants were selfed to generate F_2 plants (Fig. S1A & B). After screening of the resultant 471 F_2 plants by gene-specific primers for both transgenes, 355 plants were found to be positive for chickpea lectin, while 351 plants were found to possess an urdbean protease inhibitor in them. A pictorial representation for both is provided (Fig. 2A & B).

In the screening of all F_2 population, 270 plants were found positive for both genes either in homo or heterozygous condition; 85 were positive for CHPL but negative for UPI; 83 were positive for UPI but negative for CHPL while 33 plants were homozygous recessive for both the transgene. Looking closely into the segregation ratio, it is clearly evident that both of the P_1 and P_2 lines are single-copy transgenic for their respective genes as they assorted independently (*P*-value: 0.81) segregation in the F_2 population fitted well in 9:3:3:1 (Table 2).The observed segregation ratio is significantly closer to the expected ratio for both the transgenes. But the subtle deviation from the independent assortment might be due to segregation distortion arising due to the population size. As whole experiment was performed in pot under greenhouse conditions, also only 471 F_2 progenies survived.

Analysis of gene expression

Phloem is the main vascular tissue, always transporting sugars to various tissue of the plant;

Table 2 — Inheritance pattern of lectin and urdbean protease inhibitor transgenes in the F_2 population

Genetic combination	L_PI_	L___	__PI_	____
Observed value	270	85	83	33
Expected value if followed by 9:3:3:1	264.94	88.31	88.31	29.44
Chi-squared value	0.972			
Degree of freedom	3			
P-value	0.81			

*L_PI_, L___, __PI and ____ represent the possible allelic combinations in the F_2 population.

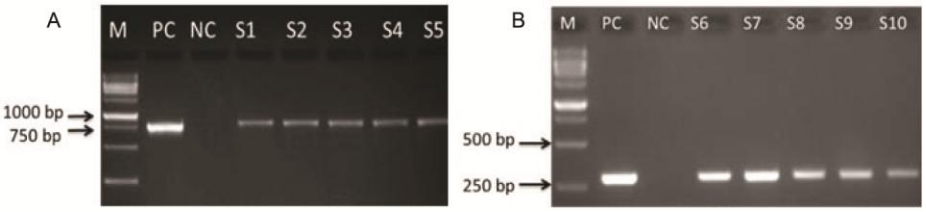

Fig. 1 — Agarose gel electrophoresis of transgenic parental lines showing the presence of (A) CHPL; and (B) UPI gene amplified from genomic DNA. M, PC, and NC represents 1000 bp DNA ladder, Positive control, and Negative control (non-transgenic), respectively, while S1-S5 and S6-S10 are P_1 and P_2individuals, respectively

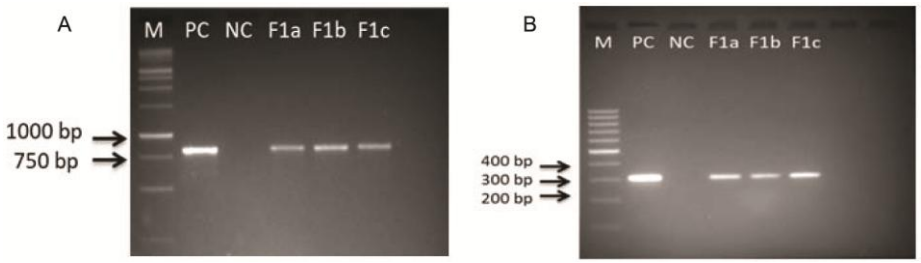

Fig. 2 — Agarose gel electrophoresis of PCR products showing the segregation of (A) CHPL; and (B) UPI gene in selected F_2 plants derived from P_1 and P_2. Lane M represent 1 Kb DNA ladder (in Fig 2A) and 100bp DNA ladder (in Figure 2B), while PC, NC represents positive control and, negative control, respectively. Lane 1-24 are randomly selected F_2 plants

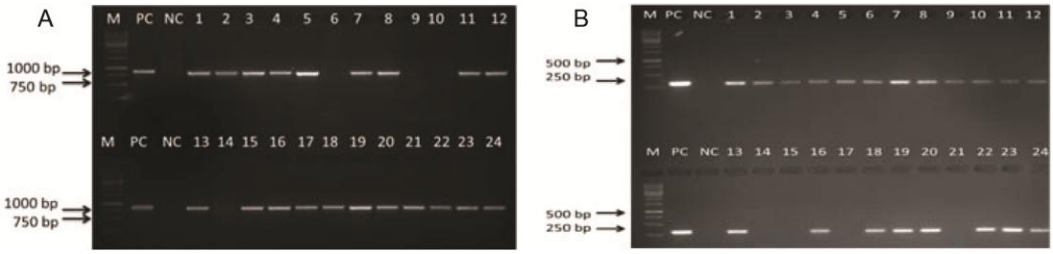

Fig. 3 — Expression analysis of CHPL and UPI gene in the leaf tissue of P_1, P_2, and pooled F_2 (10 individuals) samples before aphid infestation, 5th, and 9th day post aphid treatment

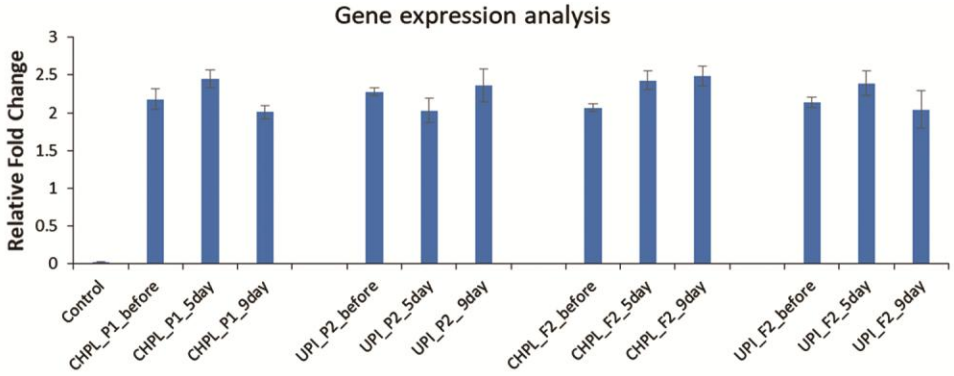

Fig. 4 — Graph showing mean percent mortality of aphid (A) (*L. erysimi)*; and (B) natality in control, P_1, P_2, and F_2 individuals over days

therefore, sucrose is always present in the phloem sap. As the *rolC* promoter is inducible by sucrose, the promoter got induced by the available sucrose and the transgene is expressed in phloem continuously. Once the aphid sucks the phloem sap, it by default also sucks the insecticidal genes present in the sap expressed under *rolC* promoter. In our study, we have taken three different stages (0, 5th and 9th day) to see the dynamics of both transgenes expression in P_1, P_2, and F_2 plants. Overall the relative fold change of both genes ranges from 1.8-2.4, as depicted from (Fig. 3). In the case of P_1, the highest gene expression (2.4 X) of the transgene was found on the 5th day post aphid infestation, followed by before (2.17 X) and 9th day post aphid infestation (1.9 X) (Fig. 3). In the case of P_2, the highest gene expression was found on the 9th day post aphid infestation (2.3 X), followed by before (2.2 X) and 5th day post aphid infestation (2.03 X) (Fig. 3). In the F_2 population, lectin gene expression was almost equivalent in the 5th and 9th days post aphid infestation (2.3 and 2.4 X, respectively), while relative fold change of the lectin was found lower before the aphid infestation (2.1 X) (Fig. 3). The UPI gene expression in F_2 population plants before and after the 9th day post aphid infestation was equivalent (1.9 and 1.8 X, respectively), while comparatively high on the 5th day (2.3 X). After consideration of the expression pattern of both transgenes, it seems that not much variation is observed in the expression pattern of both transgenes in all three stages (Fig. 3), which is an indication that the gene is expressed constitutively in phloem cells and will enter into the aphid's body once it sucks the phloem sap through phloem sap.

Aphid Bioassay

A subset of 10 plants from P_1, P_2, and F_2 populations were used, and 20 adults of *L. erysimi* were released on the 8th leaf of each plant using a clip cage. The survivability of the aphids was observed after the 3rd, 5th, 7th, and 9th days along with behavioral changes. Following 1st day, it was found that aphids feeding on transgenic plants were looking dormant, with unstable developments, oblivious and shaky movements. In control plants, aphids were healthy, growing well with proper development. Although only a few aphids were found dead on the 7th and 9th days, it might be a result of the natural defense of the non-transgenic plants.

In P_1 plants, the mortality rate was found ~30% following the 3rd day, which was increasing gradually day after day, and ~86% mortality was seen on the 9th day. Similarly, in P_2 plants, ~22.5% mortality was seen on the 3rd day and ~80% mortality was found on

Table 3 — Agronomical evaluation of control and transgenic brassica plants for yield-related traits

S. No.	Brassica plant	Plant height (cm)	Silique length (cm)	Seeds/silique	1000 Seed weight (gm)
1.	Control	120.89±1.23	6.80±0.46	10.90±0.32	7.26±0.32
2.	P_1	120.21±1.68	6.31±0.32	10.40±0.60	7.10±0.23
3.	P_2	120.46±1.73	6.02±0.49	10.35±0.57	7.23±0.16
4.	F_2	120.30±1.10	6.33±0.22	10.10±0.29	7.01±0.44

the 9th day. In the case of F_2 plants where both resistance genes from two parents were expressed together, mortality was observed to be higher, *i.e.*, ~ 41% on the 3rd day to 96% on the 9th day (Fig. 4A).

A significant increase in mortality was observed in the case of the gene pyramided F_2 population as compared to the P_1 and P_2 lines. Similar results have been reported using multiple insecticidal genes to increase the resistance of crops[31,32]. The behavioral changes in aphids were also observed, such as slow and shaky movement, looking dormant, unstable developments, unconsciousness, and avoidance of feeding with affected fecundity, which is in congruence with a previous study[27]. Therefore, the results show that the gene pyramided lines are better performing than single transgenics in terms of early mortality of aphids in pyramided lines.

The mean number of nymphs on the 9th day in control, P1, P2, and F2 individuals was found to be 215, 5, 6.7, and 2, respectively. The natality of the aphids was found to decrease significantly in the F2 individuals when compared with P1, P2, and control lines (Fig. 4B). The nymphs were found to grow in a healthy manner in the control with restricted numbers in P1 and P2 but the least number of the nymphs was found in the case of F2 plants as almost 43, 32.08, and 107.5 times decrease in the number of nymphs was found in P1, P2, and F2 individuals, respectively.

Agronomic evaluation of gene pyramided F_2 plants

A phenotypic assessment of control, transgenic P_1, and P_2, as well as the F_2 population (10 selected plants) was taken that uncovered no significant difference at the vegetative development stage. No critical adjustments were seen in plant height, silique length, seeds/silique, seed weight, and so forth, of transgenic P_1, P_2, and F_2 plants when compared with control plants under greenhouse conditions. From this, it is evident that the combination of CHPL and UPI genes in brassica didn't affect any of the morphological characteristics or crop yield. The phenotypic characteristics of the two parents, F_2 individuals, and control were phenotypically comparable (Table 3). Therefore, harnessing the legumes biodiversity using molecular markers[33,34] and their bio-prospecting[35] could be used to develop transgenics and transgenic breeding for crop improvement.

Conclusion

Among the several economically important oilseed crops, brassica is the major oilseed crop globally. In brassica, the major quality and yield losses are due to aphid infestation. To avoid the losses due to aphids, transgenic brassica has been developed containing insecticidal genes chickpea lectin (CHPL) and urdbean protease inhibitor (UPI) under the phloem specific rolC promoter separately. In this perspective, the transgenic brassica containing CHPL and UPI were crossed to develop the F_2 population. In the F_2 population, positive plants for both genes were selected through gene-specific PCR primers. The mortality of these single gene-containing transgenics against aphids was found to be 86% and 82% in P_1 and P_2, respectively, which increased to 96% in the pyramided F_2 individuals after the 9th day of aphid treatment. Further, a significant reduction of around 107.5% in the number of nymphs was observed at 9th day post aphid treatment over control. This increase in efficacy will further decrease the chance of resistance development in aphids, as the chance of aphid survival has decreased significantly once they have fed the brassica plant. Further gene expression analysis was done through qRT-PCR. The gene expression analysis did not show any significant difference before and after aphid infestation treatment in any of the samples, showing the constitutive nature of gene expression of the transgenes in phloem cells of brassica. Furthermore, no significant changes in the agronomic parameters were observed in control as well as transgenic lines, suggesting that there is no penalty attached to the transformation events.

Conflict of interest

All authors declareno conflict of interest.

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