Development of insect-resistant transgenic plants using plant genes: Expression of cowpea trypsin inhibitor in transgenic tobacco plants

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Development of transgenic plants, engineered for insect resistance, has added a new dimension to crop improvement. The cowpea trypsin inhibitor (CpTI) gene offers resistance against a wide range of insects, and has been used for developing insect-resistant plants. In the present study, the CpTI gene, amplified by PCR, was cloned in a plant expression vector under the control of the CaMV 35S promoter and the NOS terminator, and introduced into model plant tobacco by Agrobacterium-mediated transformation. The presence and expression of the CpTI gene in primary transformants R₀ and R₁ progeny was confirmed by PCR and immunobiochemical analyses. The efficacy of the expressed CpTI protein against Spodoptera litura was tested by feeding trial larvae under laboratory conditions. Reduction to the extent of 50% was observed in the biomass of S. litura larvae fed on transgenic leaves, expressing 3–5 µg CpTI/g fresh tissue.

PLANTS have several defence mechanisms against insects and other pathogens, involving defence genes whose products are detrimental to such invasions. One of these defence mechanisms involves proteinase inhibitors, which are insecticidal in nature. Serine proteinase inhibitors such as soybean trypsin inhibitor, cowpea trypsin inhibitor (CpTI), potato/tomato trypsin inhibitor (PTI) I and II, being antimeatabolic agents, have been shown to be toxic against lepidopteran insects13 and interfere in their growth and development.

The developments in genetic engineering techniques provide a means of transferring such insecticidal genes from one organism to another, including agronomically important crops, for better yield through reduced crop losses. Unlike classical breeding, this technique avoids cotransfer of undesirable genes by being selective for the target gene. Moreover, it does not pose any threat to the environment unlike many other chemical pesticide sprays. The crystal protein gene from Bacillus thuringiensis (Bt) has already been introduced in many plants such as tobacco5, tomato4, potato6, cotton7 and maize8 and has been shown to provide protection against insect pests. However Bt genes, as such, may not express as efficiently in plant systems and require extensive codon modifications to suit the plant genome9,10. In contrast, the advantage of using plant insecticidal genes over bacterial genes is that they can be introduced to other plant systems without much modification. It has earlier been shown that the transfer of proteinase inhibitor (a trypsin inhibitor) gene from cowpea11, potato12,13 and also one amylase inhibitor gene14 to tobacco or other test plants greatly reduced the damage caused by the insect pests.

In this paper, we present data on the development of transgenic tobacco plants through transfer of CpTI gene and demonstrate that the transgenic plants restrict the growth of Spodoptera litura. The CpTI gene has already been tested by other workers to be used as insecticidal gene. The gene is small (240 bp) and the absence of introns makes it easier to handle. Secondly, since the CpTI acts by binding the catalytic site of trypsin, resistance developed by insects against CpTI through mutations in the catalytic site of trypsin would be minimal.11 Also, it has been reported earlier that the gene is effective against a variety of insects such as Spodoptera litura, Heliothis virescens and Manduca sexta. The study suggests potential for optimization of the expression of CpTI to confer resistance against insect pests. To the best of our knowledge this is the first report of successful introduction of indigenously isolated and characterized CpTI gene into transgenic tobacco. The preliminary work on CpTI protein purification, antibody production, gene isolation and sequencing has already been reported15.

Standard techniques of molecular biology as described by Sambrook et al.6 were used for the construction of plasmids. The CpTI gene, earlier cloned by us in pBluescriptII KS(+) was excised using BamHI and ScaI enzymes and was subcloned in the plant expression vector pBI121 at the same sites after removal of the β-glucuronidase (GUS) gene from the vector. The construct was named as pBTI and was under the control of single CaMV 35S promoter and NOS terminator.

The resultant binary plasmid pBTI was then mobilized into A. tumefaciens LBA 4404 by triparental mating. For this, E. coli cells containing pBTI, LBA4404 cells and helper cells (containing pRK2013) were grown overnight in the LB medium containing kanamycin, rifampicin–streptomycin and streptomycin–kanamycin respectively. Cells were harvested and suspended separately in fresh LB medium (minus drug). 5 µl of each cell suspension was plated one over the other on a LA plate containing all the three drugs and allowed to grow for 2 days at 28°C. Single colonies of transformed Agrobacterium were analysed for the CpTI gene and a positive clone pBTIA was used for tobacco infection.

Transgenic plants were developed from tobacco leaf discs (Nicotiana tabacum, cv. Petit havana) by co-cultivation method17. Leaves were surface-sterilized and 5 mm discs were cocultivated for 2 h with pBTIA in MS medium with gentle shaking. Regeneration medium for shooting consisted of MS supplemented with 0.2 mg/l

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1-naphthaleneacetic acid (NAA) and 1 mg/l 6-benzylaminopurine (BAP), 100 μg/ml kanamycin and 500 μg/ml cefotaxime. After eight weeks, shoots were rooted in phytohormone-free MS medium containing 100 μg/ml kanamycin. After aqua-hardening, the regenerated plants were transferred to pots, grown in the glasshouse and self-pollinated. Mature seeds were collected and tested for germination in the absence or presence of kanamycin (150 μg/ml). Kanamycin-resistant plants were used for progeny analysis.

Total genomic DNA was isolated from R₀ and R₁ tobacco leaves according to the method described by Kochko and Hamon. Tobacco leaves (1 g) were ground in liquid N₂ and homogenized in 10 volumes of extraction buffer (50 mM tris HCl pH 8.0, 50 mM NaCl, 5 mM EDTA, 10 mM BME). 1.25% SDS was added to the homogenate and it was incubated at 65°C for 10 min. After the addition of 1.25% potassium acetate, the mixture was incubated in ice for 20 min and centrifuged at 13000 g. Precipitation of DNA was carried out by the addition of 0.5 volumes of isopropanol to the filtered supernatant and incubation at −20°C for 30 min. The nucleic acid pellet obtained after centrifugation was subjected to RNAse treatment and phenol–chloroform extraction. DNA was collected from the aqueous phase by isopropanol (0.7 vol) precipitation.

DNA, thus obtained, was tested for the presence of CpTI gene by PCR amplification using the CpTI-specific primers.

Tobacco leaf extracts were prepared according to Johnson et al. at 4°C. Leaf tissue (0.5 g) was homogenized in liquid nitrogen in an isolation buffer containing 0.1 M tris (pH 7.0), 0.5 M sucrose, 0.1% cysteine and 50 mM BME. The homogenate was centrifuged at 10000 g for 10 min and the supernatant was saturated to 50% with ammonium sulphate. The precipitate was collected by centrifuging at 2500 g for 10 min and it was suspended in 50 mM ammonium carbonate, lyophilized and resuspended in water. The brown colour of the suspension was removed by adding small amount of resin (mixed bed resin, Sigma). After centrifugation, protein was concentrated in a Speed Vac (Savant) at room temperature. This extract was used for the immuno-biochemical analyses. Proteins in the extract were estimated by Peterson's method.

Proteins from the leaf extracts of the transformed and untransformed plants were separated on 15% SDS-PAGE. Protein samples were prepared in 60 mM tris HCl pH 6.8 containing 0.1% SDS, 20 mM DTT and 0.125% bromophenol blue. Samples were boiled for 1 min before electrophoresis. After electrophoresis, the proteins in the gel were transferred on a PVDF membrane (Bio-Rad). The protein blots were first blocked with 0.5% casein, treated with anti-CpTI IgG followed by goat anti-rabbit IgG alkaline phosphatase conjugate. The blots were developed with BCIP (0.06 mg/ml) and NBT (0.1 mg/ml) in a buffer containing 0.1 M tris HCl pH 9.6, 0.1 M NaCl and 5 mM MgCl₂.

The trypsin inhibitor activity was assayed by the colorimetric method. Each reaction was carried out for 15 min at room temperature (25–26°C) using 1 μg of trypsin and α-N-benzoyl-L-arginine-p-nitro anilide (BAPNA) as substrate. The amount of nitroaniline released by trypsin with and without leaf extract in control and transgenic plants was measured spectrophotometrically (Kontron) at 410 nm. Assays were carried out using fresh leaf extracts.

Four-day-old larvae (0.5 mg/insect) of Spodoptera litura were used for feeding trials. The experiments were run in triplicates. For each set, 10 insects were placed in a beaker and fed on fresh and thoroughly washed tobacco leaves. Beakers were covered with markeen cloth to avoid escape of insects. Insects were weighed on alternate days for 10 days, changing leaves every time. Insecticide sprays were avoided for at least one month before feeding trials.

Tobacco leaves were transformed with transformation plasmids, pBl121 and pBTI. The pBl121 is an expression vector containing GUS gene whereas in pBTI, the GUS gene was replaced by the CpTI gene fragment (Figure 1). Plants transformed with pBl121 were used as

![Diagram](image)

**Figure 1.** Structure of binary plasmid constructs containing the CpTI gene. a, Binary plasmid vector pBl121; b, Binary plasmid pBTI in which the GUS fragment was replaced by CpTI gene at BamHI and SacI sites. B, BamHI; S, SacI; NOS, nopaline synthase polyadenylation region; CaMV 35S, Cauliflower mosaic virus 35S promoter; GUS, β glucuronidase gene; NPTII, Neomycin phosphotransferase gene.

![Image](image)

**Figure 2.** PCR analysis for the detection of CpTI gene in transgenic tobacco plants. Plasmid pTIVO containing CpTI gene (lane 1), total DNA from untransformed plant (lanes 2), pBl121 transformed plant (lane 3), primary transformants V₁, V₄ (lanes 4, 5), R₁ generation plants V₁-10, V₄-12, V₄-10 (lane 6–8 respectively) were analysed using CpTI gene specific primers. PCR products were separated on 1.2% agarose gel, stained with ethidium bromide and photographed under ultraviolet light.
a negative control. Twenty-five independent pBTT transformed plants were obtained on the basis of the kanamycin resistance (neomycin phosphotransferase II gene). Ten of these plants were randomly picked and tested for the presence of the CptTI gene. Using primers specific for the CptTI gene, all the ten plants showed 240 bp CptTI-specific fragment after electrophoresis of PCR amplified products. As expected no band was observed in untransformed or in pBI121 transformed plants (Figure 2). R\textsubscript{1} seeds of some of R\textsubscript{0} plants, viz. V\textsubscript{1}, V\textsubscript{2}, V\textsubscript{4}, V\textsubscript{5}, V\textsubscript{20} etc., which were obtained by selfing, were germinated in the presence of 150 mg/ml kanamycin. Seedlings were segregated in the ratio of 3:1 when selected for kanamycin (Table 1). Seedlings of untransformed tobacco seeds turned yellow in kanamycin. R\textsubscript{1} progeny of V\textsubscript{1} and V\textsubscript{4} plants also inherited the CptTI gene as indicated by PCR analysis of DNAs of these plants which showed the 240 bp band (Figure 2). Progeny of other R\textsubscript{0} plants was also analysed (data not shown) to confirm the presence of CptTI gene.

Ammonium sulphate-precipitated proteins from transformed and non-transformed tobacco leaves were electrophoresosed on a 15% SDS-PAGE and Western blots were probed with anti-CptTI IgG\textsuperscript{15}. Immunological analysis (Figure 3) showed a single band of 16.5 kD corresponding to purified CptTI protein in V\textsubscript{1} – 10, V\textsubscript{1} – 12, V\textsubscript{4} – 10 plant (V\textsubscript{1} progeny, lane 4). This band was absent in the extracts of untransformed plant CR\textsubscript{1} (lane 5) as well in plants transformed with pBI121 alone (not shown). Although the CptTI is a 8.0 kD protein in monomeric form, it exists in dimeric-multimeric forms (a characteristic of Bowman–Birk inhibitors) even under reducing conditions\textsuperscript{22}. Purified CptTI protein from cowpea also shows a molecular weight of 16.5 kD on SDS-PAGE\textsuperscript{15} in 50 mM BME. The above results indicate that CptTI, when expressed even in tobacco, retains its characteristic dimeric form.

Trypsin inhibitor activity was measured as per cent decrease in trypsin activity. We tested R\textsubscript{1} progeny of the various R\textsubscript{0} plants and it was found that the progeny of V\textsubscript{1} plants showed the highest inhibition of trypsin activity. Figure 4 shows that leaf extract from the control plants did not inhibit trypsin activity whereas leaf extracts from V\textsubscript{1} – 10 (V\textsubscript{1} progeny) and V\textsubscript{4} – 10 (V\textsubscript{4} progeny) inhibit the trypsin activity. The V\textsubscript{1} progeny by itself showed variations in CptTI inhibition. This was also indicated by different levels of CptTI accumulated in these plants. For example, V\textsubscript{1} – 10 leaves accumulated 5 µg CptTI/g fresh tissue which is higher than the CptTI protein accumulated in V\textsubscript{1} – 12 plant (3 µg/g fresh leaf). This expression is quite low when compared with the value of 50–200 µg/g tissue for other proteinase inhibitors in transformed tobacco plants driven by CaMV 35S promoter\textsuperscript{12}.

The R\textsubscript{1} progeny of V\textsubscript{1} and V\textsubscript{4} plants, which showed the presence of CptTI gene by PCR and Western blots,

Table 1. R\textsubscript{1} inheritance of the NPTII gene

<table>
<thead>
<tr>
<th>R\textsubscript{0} transgenic lane</th>
<th>KanR</th>
<th>KanS</th>
<th>χ² value (P = 0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V\textsubscript{1}</td>
<td>68</td>
<td>20</td>
<td>0.24</td>
</tr>
<tr>
<td>V\textsubscript{2}</td>
<td>78</td>
<td>22</td>
<td>0.48</td>
</tr>
<tr>
<td>V\textsubscript{4}</td>
<td>48</td>
<td>12</td>
<td>0.80</td>
</tr>
</tbody>
</table>

R\textsubscript{0} seeds were grown in the presence of 150 µg/ml kanamycin. KanR indicates R\textsubscript{1} plants carrying NPTII gene and KanS indicates plants without the NPTII gene.

Figure 3. Western blot of CptTI protein from tobacco plants transformed with the pBTT construct. Total proteins were isolated and separated on a 15% SDS-PAGE as described in text. Lanes 1–4, protein extracts from various R\textsubscript{1} generation plants, viz. V\textsubscript{1} – 10, V\textsubscript{1} – 12, V\textsubscript{2} – 8 and V\textsubscript{4} – 10 respectively; lane 5, protein extract from untransformed tobacco plant.

Figure 4. Inhibition of trypsin by tobacco leaf extracts from transgenic plants. 50% ammonium sulphate precipitated extracts were used for inhibitor studies. Activity was assayed as described in text. V\textsubscript{1} – 10, V\textsubscript{4} – 10 = R\textsubscript{1} generation plants expressing CptTI protein; C\textsubscript{1} – 7 = R\textsubscript{1} progeny of pBI121 transformed tobacco plant; C\textsubscript{–} 9 = untransformed plant.

V\textsubscript{1} – 8 plants (V\textsubscript{1} progeny, lanes 1, 2, 3) and V\textsubscript{4} – 10 plant (V\textsubscript{4} progeny, lane 4). This band was absent in the extracts of untransformed plant CR\textsubscript{1} (lane 5) as well in plants transformed with pBI121 alone (not shown). Although the CptTI is a 8.0 kD protein in monomeric form, it exists in dimeric-multimeric forms (a characteristic of Bowman–Birk inhibitors) even under reducing conditions\textsuperscript{22}. Purified CptTI protein from cowpea also shows a molecular weight of 16.5 kD on SDS-PAGE\textsuperscript{15} in 50 mM BME. The above results indicate that CptTI, when expressed even in tobacco, retains its characteristic dimeric form.
were used for insect bioassays. These experiments were repeated thrice and each experiment was run in triplicates. The results of these experiments are shown in Figure 5. The overall growth was reduced when insects were fed on positive plants (containing CpTI) in comparison to untransformed plants or plants transformed with pBI121. R₄ progeny of V₁ plants showed higher resistance than the progeny of other R₀ plants. There were variations in growth reduction and mortality in different experiments but V₁ - 10, V₁ - 12 and V₂ - 4 consistently showed better resistance. There was more than 50% reduction in biomass of the insect larvae fed on these plants compared to controls. The insects also showed a refractory tendency (i.e., going away from the leaf). Data for a typical feeding experiment is shown in Figure 6 in which a continuous change in insect biomass is shown when larvae were fed on CpTI-transformed and control tobacco leaves. The data showed that at the end of 10 days V₁ - 10 and V₁ - 12 fed larvae had only 40% and 60% weight as compared to larvae fed on leaves of control tobacco plants (Table 2). Figure 7 shows the reduction in the growth of larvae fed with the leaves of transgenic plants, while Figure 8 shows the extent of leaf damage in control and transgenic (CpTI) plants by the S. litura larvae during the same period. These data clearly indicate that the introduction of the CpTI gene in tobacco plants results in substantial reduction in the growth of insect larvae. The stability of the introduced gene was evident in the R₂ progeny (data not shown) and inheritance in the following generations is being tested.

In the present study, tobacco transgenic plants were developed carrying CpTI as a transgene. We had earlier reported cloning of the trypsin inhibitor gene from cowpea, its expression in E. coli and identification of expressed protein by Western blot analysis. The expression of the CpTI gene in the present study was driven by a single CAMV 35S promoter in the construct used for plant transformation. The presence of the gene in the regenerated plants was confirmed by PCR and Southern analysis (data not shown) whereas its expression was tested by immuno-biochemical analyses. The toxicity trials (bioassays) were conducted on the larvae of Spodoptera litura, which clearly indicated that the expressed protein was physiologically active and provided reasonable resistance. While the transgenic plants were found to be resistant to insect larvae, problems with regard to low accumulation of CpTI protein was encountered. It was, for example, observed that even the most toxic plants accumulated only 3–5 μg CpTI/g tissue or approximately 0.05% of soluble protein fraction. Hilder et al. reported high CpTI expression in their transgenic tobacco plants (1% of total soluble protein).

Table 2. Bioassay of R₁ transgenic tobacco plants against S. litura larvae

<table>
<thead>
<tr>
<th>Plant</th>
<th>Initial no. of insects</th>
<th>Initial wt per insect (mg)</th>
<th>% mortality after 10 days</th>
<th>Final wt per insect (mg)</th>
<th>% wt of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₉</td>
<td>10</td>
<td>6 ± 0.7</td>
<td>0</td>
<td>20.8 ± 2.8</td>
<td>100</td>
</tr>
<tr>
<td>C₇</td>
<td>10</td>
<td>5 ± 1</td>
<td>0</td>
<td>20.0 ± 1.6</td>
<td>96</td>
</tr>
<tr>
<td>V₁₋₁₀</td>
<td>10</td>
<td>4 ± 0.7</td>
<td>40</td>
<td>8.3 ± 0.5</td>
<td>40</td>
</tr>
<tr>
<td>V₁₋₁₂</td>
<td>10</td>
<td>5 ± 0.5</td>
<td>37</td>
<td>12.3 ± 2.3</td>
<td>59</td>
</tr>
<tr>
<td>V₂₋₄</td>
<td>10</td>
<td>4 ± 1</td>
<td>35</td>
<td>8.5 ± 3.6</td>
<td>41</td>
</tr>
</tbody>
</table>

Safed progenies of R₂ transformants were used for bioassays. Assays were carried out in triplicates. See text for details. Data shown is average of 3 assays.

Figure 5. Bioassay data for 10-day feeding trials of Spodoptera litura larvae on transgenic tobacco plants. Different bars show percent gain in larval weight when fed on transgenic and control tobacco leaves.

Figure 6. Continuous growth pattern of Spodoptera litura on a diet of transgenic tobacco leaves. Larvae were fed on leaves from untransformed plant C₉ (●●●) or leaves from V₁₋₁₀ (●●●) and V₁₋₁₂ (●●●) transgenic plants expressing CpTI protein. The insect weight shown represents the average weight per insect.
Johnson et al.\textsuperscript{12} have also reported higher levels of tomato and potato proteinase inhibitors in their primary transformants. However, it was interesting to observe that even with this low expression, our transgenic plants were able to show resistance to the larvae of \textit{S. litura}. Further modifications in the construct are being done to increase the expression of \textit{CpTI} and higher accumulation of the protein. Several reasons could be attributed for the low expression of the transgene. We have used only coding sequences of the \textit{CpTI} whereas Hilder et al.\textsuperscript{11} have added 200 bp upstream region of the gene which might be providing stability to the mRNA. Study of Masoud et al.\textsuperscript{23} on transgenic tobacco plants obtained by transferring 14 K-Cl gene (encoding corn bifunctional inhibitor) also reported low protein accumulation (5-6 \( \mu \)g/g tissue). They suggested post-transcriptional events as the major factor for the low accumulation. Methylation and gene silencing are the other phenomena which may result in the lower expression of transgenes\textsuperscript{24-26}. The gene silencing phenomenon is more pronounced when multiple copies of the foreign gene are integrated into the genome. However, in our studies, the \textit{CpTI} gene was found to be integrated only in single copy (Southern analysis, data not shown). However, the possibility of gene silencing can still not be ruled out totally. We have not tested the possibility of \textit{CpTI} gene or its promoter getting methylated. The variability in resistance shown by plants from R\textsubscript{0} to R\textsubscript{1} generation was reported earlier by others in their bioassays\textsuperscript{11}. However, it was attributed to inherently variable biological systems rather than variation in the expression.

Our main aim was to develop an indigenous system to stably transform crop plants with \textit{CpTI} gene, as ready-made constructs of \textit{CpTI} were not available. We have shown successful transformation of tobacco plant with the \textit{CpTI} gene, which will pave way to move this gene into other crop plants. Although some data on the expression of this gene in plants show that it is not as effective as some of the \textit{Bt} genes, its utility as a supplementary gene to enhance the effect of other insecticidal genes cannot be overlooked. Such an approach is desirable to restrict development of resistance by insect pests toward products of transgenes. The multigene protection strategy seems to be an attractive
Figure 8. Leaf damage of Spodoptera litura fed on leaves from transgenic plants expressing CpTII gene.
a, C-1 and C-9 control plants; b, V1-10 and V1-12 transgenic plants.

possibility and protease inhibitor, amylase inhibitor²⁷,²⁸ lectin²⁹, etc. could provide genes of choice for this purpose.

Reproductive success of large and small flies in *Drosophila bipectinata* complex

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Reproductive success of large and small flies of *Drosophila malerkotliana* and *D. bipectinata* has been studied using no-choice method. Results indicate that large males have higher remating ability and longevity than small males as a result of which they can inseminate more females in their lifetime than small males. Large females also have higher reproductive success because they have more number of ovarioles, lay more eggs and produce more fertile offspring than the small female and also mate with more males in their lifetime than small female by having higher longevity. These findings suggest that large flies have higher reproductive success than small flies.

Body size is the most obvious, easily observable and measurable phenotypic trait and there is compelling evidence indicating that it is directly related to fitness. In *Drosophila*, as in many organisms, body size is closely linked to life history traits such as fecundity, dispersal ability and mating success and has been widely used in studies on quantitative genetics. The adaptive nature of body size in *Drosophila* has also been demonstrated by many workers both in natural populations and in laboratory populations. Body size also influences mating speed, fecundity and other fitness characters.

Many workers have used thorax length as an index of body size in *Drosophila*. Apart from thorax length, other morphological traits such as wing length, wing width and face width have been used as index of body size. Wing is another phenotypic trait which can be used as an index of body size. Relationship between wing length and mating speed had been studied in *D. malerkotliana*. Correlation between copulation duration and fertility; and variation in mating propensity had also been studied in *D. bipectinata*. The fitness characters such as fecundity, fertility, male remating ability and longevity of large and small flies have not been made. Moreover, mating speed alone does not qualify an individual to have higher reproductive success. Hence the present investigation is aimed at understanding reproductive success of large and small flies in *D. bipectinata* species complex.

The stocks used in the present study were *Drosophila malerkotliana* and *D. bipectinata*. All experiments were made separately for each of these two species. The stocks used in the present study originated from 150 naturally inseminated females from Mysore, Karnataka. When progeny appeared, flies were distributed to different culture bottles and were maintained under constant temperature (22 ± 1°C). For every generation, flies multiplied in different culture bottles were mixed together and eggs were collected using Delcourt's procedure. Eggs (100) were seeded in fresh quarter pint milk bottles with 25 ml of wheat cream-agar medium to avoid larval competition during development (this procedure allows us to reduce environmental variation in size). After 10 generations, when adults emerged, virgin females and males were isolated within 3 h of their eclosion and maintained separately at 22 ± 1°C. Wing lengths of male and female flies were measured separately when they reached the required age. Each fly was etherized individually, the intact left wing kept in horizontal plane was measured from humeral cross vein to the tip with an ocular micrometer at 100 × magnification. Wing length was measured in units of 1/10 mm. After measuring the wing length, each fly was placed separately in fresh food vials to study fitness characters.

To study fecundity and fertility, five to six-day-old virgin females and male flies of chosen wing size (see Table 1 for chosen wing size) were taken and different crosses were made (large male × large female; large male × small female; small male × large female; small male × small female). Soon after mating, mated females were transferred into fresh food vials every 24 h without etherization. The total number of eggs laid in each vial and the total number of progeny appeared were counted over a period of 15 days. 50 trials were run for each cross and mean fecundity and fertility were obtained.

To study the remating ability of male, 5 to 6-day-old virgin females and male flies of chosen wing size (see Table 1 for chosen wing size) were taken and different crosses were made (large male × large female; large male × small female; small male × large female; small male × small female). When mating occurred, the pair was allowed to complete copulation. After