Results
4. RESULTS

4.1 Standardization and optimization of high throughput tomato regeneration protocol

The parameters involved in transformation of tomato were optimized for the tomato cultivar Arka Viaks, using pCAMBIA 2301 vector harboring the Arabidopsis thaliana dehydration responsive element binding protein (Atdreb1A).

4.1.1. Effect of BAP on shoot regeneration efficiency

The effect of different concentrations of cytokinin, BAP and the type of auxin IAA or IBA at 0.1 mg/L on shoot regeneration were compared. Explants cultured in IBA containing media had exhibited higher (38%) shoot regeneration compared to IAA (24.16%). It was observed that the MS medium containing BAP at the concentration ratio of 2 mg/L and 0.1 mg/L IBA gave the highest shoot regeneration 53.3% (Fig. 4.1) compared to other BAP concentration used (Fig. 4.5A). The other concentration of BAP examined at the concentration of 3 mg/L, 4 mg/L and 5 mg/L BAP gave 41.80%, 38% and 41.37% respectively, showed almost similar shoot regeneration percentage, BAP at 1 mg/L gave lowest regeneration percentage of 24.50%.

![Bar graph showing shoot regeneration percentages at different BAP concentrations](image)

**Figure 4.1:** Shoot regeneration percentages at different BAP concentrations in shoot induction. (Error bar represents standard error mean (± SEM) for five replicates).
4.1.2 Influence of age of seedlings

The influence of age of the seedlings were examined on the shoot regeneration percentage obtained from 8th, 9th and 10th day were 38.5, 40.1 and 48.2 respectively, under the supplementation of BAP at 2 mg/L. Statistically, 8th and 9th day old seedlings exhibited similar regeneration response, but the 10th day old seedlings were significant (p>0.05) over 8th and 9th day (Fig. 4.2). The 10th day old seedlings were used for the development of transgenic plants.

![Influence of age of seedlings](image)

**Figure 4.2:** Shoot regeneration percentages at different aged seedlings in shoot induction. (Error bar represents standard error mean ± SEM for five replicates).

4.1.3 Effect of explant type on shoot regeneration efficiency

In this study, cotyledon and hypocotyl explants were used to compare shoot regeneration efficiency. After co-cultivation and elimination of *Agrobacterium* in SRM medium, the explants showed regeneration response by swelling and producing callus within 10 days. Hypocotyl explants exhibited higher (53.3%) shoot regeneration than cotyledons (22.8%) on the SRM. Statistically, hypocotyls exhibited superior regeneration over cotyledon (p>0.05). This difference in the shoot regeneration response of explants is due to a low realization of shoot buds into elongated shoots in cotyledons (Fig. 4.5A & B), even though cotyledons had given higher number of shoot buds individually, in hypocotyls the realization of shoot buds into elongating healthy shoots was higher, whereas, most of the shoots formed on cotyledons were rosette-like leafy structures, which showed difficulty in elongation.
Figure 4.3: Shoot regeneration percentage from cotyledon and hypocotyl explants. (Error bar represents standard error mean ± SEM for five replicates).

4.1.4 Influence of *Agrobacterium* cell density on shoot regeneration

The influence of *Agrobacterium* cell density in tomato transformation was evaluated. The *Agrobacterium* cell density OD$_{600nm}$ at 0.1, 0.25 and 0.5 were given different shoot regeneration response. The shoot regeneration was obtained highest for 0.1 OD with 38.16% and for other cell densities 18.33% and 14.33% respectively (Fig. 4.4). Statistically the shoot regeneration obtained at 0.1 OD$_{600nm}$ was significant over other two tested *Agrobacterium* cell densities (p>0.05).

Figure 4.4: Shoot regeneration at different *Agrobacterium* cell density. Error bar represents standard error mean for five replicates.

4.1.5 Effect of auxins on rooting of the regenerated shoot

The regenerated and elongated shoots (Fig. 4.5C) of tomato rooted readily in presence of auxin. In this experiment, the influence of the auxin source IAA or IBA on rooting of the regenerated tomato shoots was evaluated. It was observed that
highest rooting of 66.6% was occurred in 0.5 mg/L IBA compared to 29.16% in IAA within 21 days of incubation in rooting medium (Fig. 4.5D). The rooted plants were hardened (Fig. 4.5E) and maintained at biosafety net house (Fig. 4.5F).

4.1.6 Duration of transformation and regeneration

The period required for the shoot regeneration and rooting in the transformation of tomato was recorded (Table 4.1). The shoot bud formation started at 12 days and the shoot regeneration response to obtain a shoot length of 8-10 mm was around 24 days in (34%) and regeneration rates (up to 53.2%) in a short period of 62 to 77 days, in the hypocotyls. In hypocotyl explants 82.44% of shoot regeneration was obtained within 4 to 5 weeks. In the present study, the time frame for tomato transformation was about two and half months which is comparable to that of tomato cultivar Rio Grande by Khoudi et al. (2009), but considering the antibiotic selection medium which drastically reduces the transformation rate and efficiency, the present experiment achieves high transformation antibiotic selection medium without the use of complicated tobacco or tomato feeder layer or acetosyringone.

Table 4.1: Comparison of time duration of different tomato transformation protocol

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<tr>
<td>Preparation (d)</td>
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<td>Preculture (d)</td>
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<td>Co-cultivation (d)</td>
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<td>Agrobacterium elimination (d)</td>
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<td>Shoot induction (d)</td>
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<td>21-28</td>
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<td>Shoot elongation (d)</td>
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<td>Rooting (d)</td>
<td>21-28</td>
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<tr>
<td>Transformation period (d)</td>
<td>3-4 months</td>
<td>2-3 months</td>
<td>2-2.5 months</td>
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</table>
Figure 4.5: *Agrobacterium* mediated shoot regeneration of tomato. A & B) are shoots regenerated from hypocotyl and cotyledon explants, C) Regenerated shoots in elongation medium, D) Rooting of the elongated shoots in rooting media, E) Acclimatization of rooted tomato plants in polythene bags, F) Maintenance of transgenic plants at biosafety net house facility.
4.2 Gene constructs preparation

In this objective, H. armigera target genes HaSP and HaJHAMT were used for the ihpRNA forming gene construct for the development of transgenic plant. The gene constructs prepared in pGRNAi for plant transformation were verified and used for plant transformation of tomato.

4.2.1 Selection of H. armigera target gene

Two target genes of H. armigera sequences were chosen based on the preliminary bioassay in vitro reported by Asokan et al. (2012) at Indian Institute of Horticultural Research (IIHR). HaSP gene fragment of size 500 bp from +191 to +690 was isolated using the gene specific primers (Table 4.2). Similarly, HaJHAMT gene fragment of size 500 bp from +193 to +692 was isolated. The selection of the fragment size was based on off-target minimized dsRNA sequence as predicted by the dsCHECK software.

The minimization of the off-target effect is of paramount importance in conducting any RNAi experiments. The lack of specificity may lead to unintended effects. For this reason the nucleotide sequence was searched for extent of sequence similarities in the NCBI database, the search targets were likely the parasites and predators of H. armigera and beneficial insects, wherever the sequence information was available. The selected region of the H. armigera HaSP fragment was evaluated for sequence similarity search aligned with HaSP gene of A. mellifera, B. mori, Homo sapiens and they were compared for minimum sequence homology (Fig. 4.6). It was found that there was no significant sequence homology above 17 nucleotides, similarly in case of HaJHAMT, the sequence was aligned with insects A. mellifera, S. litura, B. mori and A. pismum for HaJHAMT homology, it was observed that there was no significant sequence homology above 15 nucleotides (Fig. 4.7). Further the 500 bp fragments of both HaSP and HaJHAMT were searched using ntBLAST with the host plant tomato and A. thaliana where complete genome sequence data are available but no significant sequences similarities were found within the genomes.
Figure 4.6: The multiple sequence alignment of HaJHAMT with its parasites and predators of H. armigera.
Figure 4.7: The multiple sequence alignment of HaSP with its parasites and predators of H. armigera.
4.2.2. Preparation of gene cassettes for plant transformation in pGRNAI vector

The above two target genes of *H. armigera* viz., *HaSP* and *HaJHAMT* were PCR amplified to incorporate restriction enzyme cleavage sites and were subcloned into modified pBluescript II KS+ vector. Two introns were utilized, 1\(^{st}\) intron from *Psy1* gene of tomato and for intron mediated ihp a *Pds 6\(^{th}\)* intron was used with optimized synthetic splice sites. The use of two introns ensures a clean dsRNA (of target gene) without significant vector sequence contamination.

4.2.2.1. Cloning of Phytoene synthase in pBluescript II SK+

To express the target dsRNA in tomato without contaminating vector sequences to avoid any cross homology and the consequent off target effects, the strategy was designed in such way that the expressed RNA transcript snaps back and forms dsRNA in plant system. For this purpose first intron of *Psy1* (140bp) having splicing site of tomato was PCR amplified (Fig. 4.8) using genomic DNA of tomato and was ligated at the 5\(^{\prime}\) region of the target *HaSP* and *HaJHAMT* genes. An enzyme recognition site i.e. *SacII* forward and *BamHI* reverse was incorporated to PCR amplification of *psy1*. The PCR amplified product was restriction digested with above enzymes and cloned into the pBluescript II SK+.

![Figure 4.8: Cloning of 1\(^{st}\) intron of *Psy1* gene with specific primers containing restriction sites. Lanes: M-Hyper ladder V, 100-500 bp; 1-4 are PCR products of *Psy1* intron (140 bp).](image)
4.2.2.2. Cloning of HaSP and HaJHAMT in pTZ57R/T

The *HaSP* and *HaJHAMT* gene fragment was amplified using primers incorporating RE sites i.e. for *HaSP*, *BamHI* and *SalI*, and for *HaJHAMT*, *BamHI* and *HindIII* were used. The amplified PCR product of 500 bp size was confirmed in both *HaSP* and *HaJHAMT* gene by agarose gel electrophoresis (Fig. 4.9).

![Figure 4.9: PCR amplification of serine protease and juvenile hormone acid methyl transferase. Lanes: M-Hyper ladder I, 0.2-10Kb; Lanes HaSP and HaJHAMT PCR products (500 bp).](image)

The PCR amplified products of *Psy1*, *HaSP* and *HaJHAMT* were purified by gel elution. *HaSP* and *HaJHAMT* were cloned in pTZ57R/T and *Psy1* was cloned in pBluescript II SK+.

![Figure 4.10: Plasmid clones of Psy1 intron in pBluescript II SK+, HaSP and HaJHAMT sequences in pTZ57R/T. Lanes: M-EcoRI-HindIII double digested λ DNA; Lane 1- untransformed control; 2-4 Psy1 intron; 5-8 are HaSP 500bp clones and 9-12 are HaJHAMT 500 clones.](image)
The transformed colonies containing the recombinant plasmids were confirmed for the presence of insert by observing size variations in agarose gel (Fig. 4.10). Further, the presence of the 500 bp gene fragment of HaSP and HaJHAMT insert in the pTZ57/R vector was individually confirmed by RE digestion with respective RE’s (Fig. 4.11). The released product was used to clone in pBluescript II SK+ containing Psyl intron.

![Image](image_url)

**Figure 4.11:** Restriction digestion of HaSP & HaJHAMT plasmid clones (pTZ57R/T) to release the 500 bp HaSP and HaJHAMT fragments (lanes 5-6). Lanes: M-Hyperladder-I, 0.2 -10 kb; 1-uncut pBluescript II SK+ control plasmids; 2-3 are linearized pBluescript II SK+ plasmid containing Psyl intron; 4-plasmid transformed with SP insert.

4.2.2.3. **Sub cloning of HaSP and HaJHAMT fragments in pBluescript II SK+ containing Psyl**

Intron was ligated to the 5’ end of the DNA fragments of HaSP and HaJHAMT genes individually by RE digestion of pBluescript II SK+ containing Psyl gene with BamHI and SalI for HaSP and BamHI and HindIII for HaJHAMT respectively. Similarly, restriction enzyme digested and gel purified target gene fragments were ligated to splice the intron. After ligation, bacterial transformation was performed and the plasmid clones with expected size were selected (Fig. 4.12). The recombinant plasmid contained 5’ Psyl intron with HaSP and HaJHAMT fragments were confirmed by RE digestion which released the 664 bp of HaSP and HaJHAMT insert (Fig. 4.13).
Figure 4.12: Plasmid clones containing *Psy1:HaSP* and *Psy1:HaJHAMT* fragments of *HaSP* and *HaJHAMT* clones. Lanes: M-EcoRI-HindIII double digested λ DNA; 1-untransformed control; 4 & 6 are *HaSP*; 10, 14 & 15 are *HaJHAMT*.

Figure 4.13: Confirmation of the recombines by RE digestion and insert release of *Psy1*intront: *HaSP* and *Psy1*: *HaJHAMT* fragment containing clones. Lanes: M-Hyperladder-I, 0.2 -10 kb; 1- *Psy1*intront: *HaSP*; 2- *Psy1*: *HaJHAMT*.

4.2.2.4 Assembling RNAi forming cassette in modified pBluescript vector

In order to assemble gene cassette for producing the inverted repeats of *HaSP* and *HaJHAMT* gene fragments with *Psy1* intron at their 5’ region interspersed by the *Pds* 6th intron which is inbuilt in the modular vector (pGRNAi) and is flanked by sense and antisense MCS sites. To release the *Pds* intron single restriction enzyme digestion was carried i.e. with *SalI* for *HaSP* and *HindIII* for *HaJHAMT* respectively (Fig. 4.14). The released respective RE site containing introns were ligated with
*HaSP* (664bp), *HaJHAMT* (664bp) separately into modified pBluescript vector, which was previously digested with *SacII* (Fig. 4.14) and was dephosphorylated to minimize the self-ligation of the vector. For ligation reaction 1:2:1 molar ratio of vector: insert: intron was used. The transformed colonies were checked for the expected insert size (Fig. 4.15) of complete

**Figure 4.14:** RE digestion of modified pBluescript vector for ihp assembly of individual *HaSP* and *HaJHAMT* fragments. Lanes: M- Hyper ladder I, 0.2-10 Kb; 1- modified pBluescript vector cut with *SacII*; 2- modified pBluescript vector cut with *HindIII* to release *Pds* intron; 3- modified pBluescript vector cut with *SalI* to release *Pds* intron and 4- as uncut modified pBluescript vector.

**Figure 4.15:** Modified bluescript vector plasmid clones containing the inverted repeats of *HaSP* and *HaJHAMT* fragments flanked by two introns. Lanes: M- *EcoRI*- *HindIII* double digested λ DNA; 1- untransformed control; 4 & 6 are *HaSP*; 10, 14 & 15 are *HaJHAMT*.
inverted repeats *Psy*I intron: sense. *HaSP* and *HaJHAMT* cassette of 1.7 kb was released by RE digestion with *Asc*I enzyme to confirm the insert (Fig. 4.16). The released product was gel eluted and used to clone in pGRNAi vector.

Figure 4.16: Single RE digestion confirmation of *HaSP* and *HaJHAMT* inverted repeats (IR) (1.7 kb). Lanes; M-Hyperladder-I, 0.2 -10 kb; 1- Uncut; 2-*HaSP* IR; 3&4 *HaJHAMT*; 5-Uncut modular pGRNAi vector; 6-*Asc*I cut modular vector.

4.2.2.5. Cloning of *HaSP* and *HaJHAMT* ihp forming cassette in pGRNAi

To insert *HaSP* and *HaJHAMT* inverted repeat cassette into the pGRNAi vector, the pGRNAi vector was RE digested with *Asc*I (Fig. 4.16, Lane no 6).

Figure 4.17: Plasmid clones of pGRNAi carrying the SP inverted repeats with two flanking introns for mobilization into *Agrobacterium* for plant transformation. Lanes: M- *Eco*RI- *Hind*III double digested λ DNA; Lane 1, 3, 4, 5 & 7 are *HaSP* clones, 2, 6 & 8 are untransformed clones.
The linearized vector was purified, dephosphorylated to avoid self-ligation. The *AscI* cut released ihp forming cassette of 1.7 kb of *HaSP* and *HaJHAMT* fragments were ligated to the pGRNAi vector. The transformed colonies were confirmed for the recombinant plasmid (Fig. 4.17 and Fig. 4.18).

![Image of gel electrophoresis](image)

**Figure 4.18:** Plasmid clones of pGRNAi carrying the *HaJHAMT* inverted repeats with two flanking introns for mobilization into *Agrobacterium* for plant transformation. Lanes: M-EcoRI-HindIII double digested λ DNA; Lane 1, 2, 3, 4, 5, 8 & 9 are *HaJHAMT* clones, 6 and 7 are untransformed clones.

4.2.2.6. **Confirmation of *HaSP* and *HaJHAMT* insertion in pGRNAi modular vector**

![Image of gel electrophoresis](image)

**Figure 4.19:** Confirmation of ihpRNA forming cassette in pGRNAi plant transformation vector by RE digestion. M-Hyper ladder 1.0.2-10kb; 1 and 3 are *HaSP* uncut clones; 2 and 4 are RE digested pGRNAi-*HaSP* releasing 1.7 kb insert of *HaSP*; Lane 5 and 7 are *HaJHAMT* uncut clones; 5 and 7 are RE digested pGRNAi-*HaJHAMT* releasing 1.7 kb insert of *HaJHAMT*.

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Further the presence of the 1.7 kb insert was confirmed by RE digesting with *Ascl* enzyme which confirmed the insert in the plant transformation vector (Fig. 4.19).

4.2.2.7. Confirmation of orientation of antisense gene of *HaSP* and *HaJHAMT* in pGRNAi.

The orientation of the assembled fragments in the recombinant plasmid developed for ihpRNA expression of *HaSP* and *HaJHAMT* were further confirmed for sense and antisense fragments using gene specific reverse and AOX reverse primers. The transformed plasmid confirmed the amplification of 700 bp product by PCR for both the fragments for antisense orientation (Fig. 4.20).

![Figure 4.20](image)

**Figure 4.20:** Confirmation of orientation of antisense gene of *HaSP* and *HaJHAMT* in pGRNAi vector using gene specific reverse and AOX reverse primers. PCR product of 700 bp was obtained. Lanes: M-Hyperladder-I, 0.2 -10 kb; 1 & 2 are *HaSP* PCR product; 3 & 4 are *HaJHAMT* PCR products.

4.2.2.8. Mobilization of pGRNAi- *HaSP* and pGRNAi- *HaJHAMT* into *Agrobacterium tumefaciens* EHA105 by electroporation.

pGRNAi-*HaSP* and pGRNAi-*HaJHAMT* vector were mobilized into *Agrobacterium tumefaciens* strain EHA105 by an optimized electroporation protocol. Clones were selected on the dual antibiotic selection media, viz., kanamycin 50 µg /mL tetracyclin 10 µg/mL. The clones were further confirmed by colony PCR (Fig. 4.21) and sequencing. The mobilized *Agrobacterium* cells were used for tomato plant transformation.
Results

Figure 4.21: Colony PCR for confirmation of pGRNAi-HaSP and pGRNAi-HaJHAMT in Agrobacterium cells using gene specific reverse and AOX reverse primers. PCR product of 700 bp was obtained. Lanes: M-Hyperladder-I, 0.2 -10kb; 1-plasmid +ve control; 2-7 HaSP PCR product; 8- plasmid +ve control; 9-14 are HaJHAMT PCR product.

4.3. Development of Transgenic RNAi plants using Agrobacterium mediated transformation of tomato

The regeneration protocol optimized in the first objective was used in the development of tomato transgenic plants. The gene construct prepared in pGRNAi vector harboring the two separate genes i.e., pGRNAi-HaSP and pGRNAi-HaJHAMT as developed in the objective II was used for the Agrobacterium mediated transformation. For the development of transgenic plant a hormone concentration of 2 mg/L BAP and 0.1 mg/L IAA was used as supplements in MS medium. The regeneration study was further extended to determine the regeneration potential among hypocotyl segments and influence of cotyledon orientation using two pGRNAi gene constructs carrying SP and chymotrypsin (HaCHY) gene in Agrobacterium strain EHA 105. The hypocotyl explants were prepared by dissecting it transversely into three segments (Fig. 4.22) HYP-seg I (proximal), HYP-seg II (middle) and HYP-seg III (distal) and the regeneration from each of these groups was monitored. Interestingly, there was a significant difference in shoot regeneration response was observed among the hypocotyl segments. Overall, the pGRNAi-HaSP and pGRNAi-HaCHY transformed HYP-seg I showed similar and highest shoot regeneration percentage compared to other two segments. The regeneration percentage of HYP-seg-I obtained in both the construct was 28.65% and 23.23%. Similarly, in HYP-seg
**Results**

II transformed had 11.86% & 10.79% and for HYP-seg III was 11.20% and 11.61% respectively, (Fig. 4.22A&B). The overall regeneration response of HYP-seg I was significantly higher (p>0.05) than the other two segments, the HYP-seg II and the HYP-segIII, which did not exhibit significant variations in regeneration response in independent experimentation. However, maximum rate of shoot regeneration was obtained in the Hyp-seg I in both the vectors transformed.

**Figure 4.22:** Shoot regeneration efficiency of the hypocotyl type. A &B) Shoot regeneration efficiency was recorded highest for Hyp-seg-I compared to other two type hypocotyl segments in two vector transformed and was statistically significant (p>0.05) (Error bar represents standard error mean ± SEM).

The influence of orientation of cotyledon on shoot regeneration evaluated. The cotyledons were inoculated in both, abaxial and adaxial orientations on separate culture dishes containing MS medium supplemented with BAP 2 mg/l and 0.1 mg/l IAA in the antibiotic medium. The cotyledon inoculated with abaxial surfaces had given highest regeneration percentage (20.83%) as compared to adaxial surface (10.89%) (Fig. 4.23), the results were statistically significant (p>0.05). In the antibiotic selection medium, the cotyledon expanded in size and was green in color. The initiation of the callus during the first week and most of them completed callus formation by second week. The initiation of the shoot buds started at the cut end of the cotyledons on 10th day and continued for 28 days.
Figure 4.23: Influence of cotyledon orientation on the shoot regeneration efficiency. The highest regeneration efficiency was observed in abaxial orientation in contrast to adaxial orientation and were statistically significant (p>0.05) (Error bar represents standard error mean ± SEM).

Figure 4.24: Regeneration response of hypocotyl and cotyledon explants. A) Different hypocotyl type segments displayed a regeneration response in antibiotic selection medium. B) Adaxial and C) abaxial orientation of the cotyledon in the MS medium and its regeneration response D) The rooting of the excised shoots in the rooting medium.
Figure 4.25: Transgenic tomato plants developed using pGRNAi-HaSP and pGRNAi-HaJHAMT vector. A&B) Shoot regeneration from hypocotyl and cotyledon, C) Shoots were rooted in rooting medium, D&E) The putative transgenic plants are hardened in plastic bags and then were shifted to pots and maintained in transgenic biosafety net house.

The cotyledons that were placed adaxially in the antibiotic selection medium were curved and turned into cup shaped structures (Fig. 4.24C), whereas the abaxially inoculated explants appeared dome shape in the center and some were completely flat in structure (Fig. 4.24B). However, morphogenetic potential of the abaxially placed cotyledon was higher compared to adaxial orientation. A total of 40 pGRNAi-HaSP and 50 pGRNAi-HaJHAMT transgenic plants were developed. All the transgenic plants were maintained in the biosafety transgenic poly house (Fig. 4.25) and were screened for transgene integration.
4.4. Molecular analysis of pGRNAi-\textit{HaSP} and pGRNAi-\textit{HaJHAMT} primary transgenic plants

The primary transformants of \textit{HaSP} and \textit{HaJHAMT} dsRNA expressing plants were screened for the presence of transgene using PCR and transgene integration was confirmed by Southern blotting. The single copy events were further advanced to T\textsubscript{1} generation and segregation analysis was performed using Chi square test.

4.4.1 PCR analysis of the T\textsubscript{0} tomato plants

The acclimatized T\textsubscript{0} tomato plants growing in the polyhouse were analyzed for the presence of transgene by PCR. Two sets of primers were used, one set with vector specific \textit{nptII} gene and \textit{nos} promoter for rapid screening of both the \textit{HaSP} and \textit{HaJHAMT} putative transgenic plants, another set with 35SCaMV and gene-specific primers for the amplification of gene cassette. The primers sequences are listed in the Table 4.1.

The vector specific primers \textit{nptII} and \textit{nos} promoter was used for rapid analysis of all the transgenic plants. An amplicon size of 1.1 kb proved the transgene status of the tomato plants. (\textbf{Fig. 4.26} and \textbf{Fig. 4.27}).

\textbf{Figure 4.26:} PCR amplification of the T\textsubscript{0} pGRNAi-\textit{HaSP} transgenic plants. The transgenic plants confirming the amplification of 1.1 kb PCR product. Lanes: M-Hyperladder- I, 0.2-10kb; NTC- No template control; +ve plasmid positive control.
Figure 4.27: PCR amplification of the T₀ dsRNA expressing pGRNAi-\textit{HaJHAMMT} transgenic plants. The transgenic plants confirming the amplification of 1.1 kb PCR product. Lanes: M-Hyperladder-I, 0.2-10kb; NTC- No template control; +ve plasmid positive control.

Further, the selected PCR amplified transgenic plants of pGRNAi-\textit{HaSP} and pGRNAi-\textit{HaJHAMMT} were examined by PCR analysis with 35SCaMV primer and gene specific \textit{SP} and \textit{JHAMMT} reverse primer. It was observed that a band of expected size (1.4kb) was amplified (Fig. 4.28).

Figure 4.28: The PCR amplification of 1.4kb gene fragment of T₀ dsRNA expressing transgenic \textit{HaSP} and \textit{HaJHAMMT} plants. Lanes: M-Hyper ladder I, 0.2 to 10kb; lane 1, 2, 5 and 6 are \textit{HaSP} transgenic plants; lane 11 and 12 are \textit{HaJHAMMT} transgenic plants; -ve-non-transgenic plant; NTC- No template control; +ve plasmid positive control.
4.4.2. Confirmation of the *HaSP* and *HaJHAMT* gene expression by Reverse transcriptase PCR.

The primary transgenic plants confirmed in T₀ were analyzed for the expression of dsRNA by reverse transcription polymerase chain reaction (RT-PCR). The transgenic pGRNAi-*HaSP* plants such as SP3, SP9 and SP15 have amplified the expected size of 500 bp in PCR (Fig. 4.29). Similarly, the transgenic plants of pGRNAi-*HaJHAMT* line no JH2 and JH40 also confirmed in the RT-PCR which amplified 500 bp *HaJHAMT* gene from the plants (Fig. 4.30).

![Image](image_url)

**Figure 4.29:** RT-PCR confirmation of *HaSP* gene in transgenic tomato plants. Lanes: M- Hyper ladder -1 (0.2-10 kb); 1 +ve control; 2- SP3 and 3- SP9 & 4- SP 15(500 bp).

![Image](image_url)

**Figure 4.30:** RT-PCR confirmation of *HaJHAMT* gene in transgenic tomato plants. Lanes: M- Hyper ladder-1 (0.2-10 kb). Lanes; 1-JH2, 2-JH40; NTC-No template control; +ve- plasmid positive control.
4.4.3. Transgene integration by Southern blotting

To confirm the stable genomic integration of the transgene, all the dsRNA expressing \( T_0 \) tomato transgenic plants raised for insect target gene silencing *viz.*. *HaSP* and *HaJHAMT* were analyzed by Southern blotting. Probe for Southern analysis was prepared by labeling a 1.1 kb length of DNA segment by PCR using the Digoxigenin labeled 11-dUTP. The DIG labeled probe was evaluated by visualizing 2 \( \mu \)L of probe and control in the agarose gel (Fig. 4.31). The labeled and unlabeled PCR products (control) had a shift in the electrophoretic mobility, as the DIG labeled PCR product has a significantly greater molecular weight than that of the unlabeled. This labeled product was directly used for hybridization.

![Image](image.png)

**Figure 4.31:** Evaluation of the labeling efficiency of the PCR-labeled probes by agarose gel electrophoresis. Lanes: M-Hyper ladder I, 0.2-10kb; lane 1- DIG labeled *nptII* probe (1.3Kb); lane 2- *nptII* PCR product (1.1Kb).

For Southern analysis about 10 \( \mu \)g of genomic DNA was used for digestion with *HindIII* restriction enzyme, the digested DNA sample on running in the agarose gel electrophoresis (Fig. 4.32) showed a complete DNA digestion as a uniform smear indicating successful restriction digestion of the genomic DNA. Hybridisation of the probe to the blotted membrane showed signals on exposure to chemi-luminescence detector. Most of the transgenic plants showed positive signal, except for the wild type control Arka Vikas which served as a control and confirmed positive for gene integration (Fig. 4.33).
Figure 4.32: Restriction digestion of the genomic DNA for Southern blotting. Lanes: M-\textit{Hind} III double digests; Control-Wild type; \textit{HaSP} and \textit{HaJHAMT} transgenic samples.

Figure 4.33: Southern hybridization analysis of pGRNAi-\textit{HaSP} and pGRNA-\textit{HaJHAMT} transgenic plants. Lanes: +ve-plasmid positive control; control is the non-transformed tomato genomic DNA; SP3, SP9, SP15 are \textit{HaSP} transgenic plants; JH2 and JH40 are the \textit{HaJHAMT} transgenic plants.
The serine protease transgenic lines i.e. SP3, SP9 and SP15, the transgenic *HaJHAMT* lines JH2 and JH40 showed the single copy transgene integration in the genome. The confirmed single copy transgenic lines were advanced for T₁ generation for the homozygous elite line selection for the insect bioassay.

### 4.4.4. Segregation analysis of T₁ transgenic lines by progeny testing

The single copy *HaSP* and *HaJHAMT* transgenic lines were advanced to the T₁ generation. Seeds collected from primary transformants were sown in the pro-trays and T₁ seedlings were raised (Fig. 4.34), at this stage DNA was isolated (Lodhi *et al.*, 1994) and the plants were screened for the presence or absence of transgene by PCR amplification using *nptII* primers (Fig. 4.35 and Fig. 4.36). The PCR results showed segregation of one by third population with insert and rest without the insert, indicating the Mendelian monogenic segregation ratio of 3:1 and also showed the single copy stable gene integration in the genome (Table 4.2).

![Figure 4.34: T₁ generations of *HaSP* and *HaJHAMT* transgenic seedlings in the pro-tray.](image-url)
Figure 4.35: Progeny testing of pGRNAi-HaSP transgenic plants by PCR. The transgenic Serine protease plants amplified 1.1 kb PCR product and confirmed for the transgene in the plant. Lanes: M-Hyper ladder marker I, 0.2-10kb; lane 1-10 are SP3 progenies; 11 to 21 are SP9 progenies and 22 to 32 are SP15 progenies; –ve and +ve are non-transformed plant and positive plasmid control.

Figure 4.36: Progeny testing of pGRNAi-HaJHAMT transgenic plants by PCR. The transgenic HaJHAMT plants amplified 1.1 kb PCR product and confirmed for the transgene in the plant. M-Hyper ladder marker I, 0.2-10kb; lane 32-43 are JH40 progenies; 44 to 50 are JH2 progenies; 51 to 62 are pGRNAi vector control progenies; –ve and +ve are non-transformed plant and positive plasmid control.
The results of progeny test correlated the Southern results obtained in $T_0$ generation. The chi square test analysis showed its non-significance at **$p>0.05$ and confirmed the single copy integration as assessed by the Southern analysis and the monogenic pattern of Mendelian segregation pattern. The selected lines were analyzed for the level of the transgene expression using the Northern analysis.

**Table 4.2:** PCR segregation analysis of pGRNAi-\textit{HaJHAMT} and pGRNAi-\textit{HaSP} transgenic plants using chi square tests. The results depicted with ** lines showing 3:1 ratio of segregation.

<table>
<thead>
<tr>
<th>Transgenic lines selected</th>
<th>No of plants PCR +Ve</th>
<th>No of plants PCR -Ve</th>
<th>Total no of plants</th>
<th>Chi-square</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP3</td>
<td>8</td>
<td>2</td>
<td>10</td>
<td>0.1333**</td>
</tr>
<tr>
<td>SP9</td>
<td>9</td>
<td>2</td>
<td>11</td>
<td>0.0681**</td>
</tr>
<tr>
<td>SP15</td>
<td>6</td>
<td>4</td>
<td>10</td>
<td>0.3**</td>
</tr>
<tr>
<td>JH 2</td>
<td>7</td>
<td>0</td>
<td>7</td>
<td>0.583</td>
</tr>
<tr>
<td>JH40</td>
<td>8</td>
<td>4</td>
<td>12</td>
<td>0.0681**</td>
</tr>
</tbody>
</table>

**4.4.5. Northern analysis of transgenic RNAi plants for dsRNA expression**

The expression level of the \textit{HaSP} and \textit{HaJHAMT} dsRNA in transgenic tomato plants were examined using Northern hybridization.

**4.4.5.1. Northern analysis of transgenic pGRNAi-\textit{HaSP} plants**

The Southern confirmed transgenic \textit{HaSP} plants were screened to determine the dsRNA expression level. All the SP3, SP9, SP15, wild-type and vector control plants were also included for Northern analysis (\textbf{Fig. 4.37}). The transgenic plants SP15 and SP9 showed highest and in SP3 lowest expression levels were observed, signals for expression were detected neither in wild type nor in vector control.
Results

<table>
<thead>
<tr>
<th>Control</th>
<th>Transgenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>W VC</td>
<td>SP15 SP9 SP3</td>
</tr>
</tbody>
</table>

**Figure 4.37:** Northern blot for quantitative analysis of *HaSP* dsRNA transcripts from the leaves of different transgenic and non-transgenic plants. The upper lane indicating the Northern blot signals, i.e., Wild-type(W), vector control (VC), SP15, SP9 and SP 3 leaves, the lower lanes indicating a total RNA as a loading control.

### 4.4.5.2. Northern analysis of transgenic pGRNAi-*HaJHAMT* plants

An expression level of dsRNA of *HaJHAMT* in the T2 tomato lines was assessed by Northern analysis. Ten T2 progeny of two selected T1 lines, JH2A and JH2B were analyzed (**Fig. 4.38**). Two transgenic plants JH2A-2 and JH2B-6 had the higher expression and rest of the plants expressed moderately. The expression was absent in both wild type and vector control plants.

<table>
<thead>
<tr>
<th>Transgenic lines</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 2 3 4 5 6 7 8 9 10</td>
<td>W VC</td>
</tr>
</tbody>
</table>

**Figure 4.38:** Northern blot for quantitative analysis of *HaJHAMT* dsRNA transcripts from the leaves of different transgenic and non-transgenic plants. The upper lane indicating the Northern blot signals, Lanes; 1-JH2A-1, 2- JH2A-2, 3- JH2A-4, 4-JH2B-5, JH2B- 6, JH2B-7, JH2B- 8, JH2B- 9, JH2B- 10, JH2B- 11, W and VC are wild-type and vector controls, the lower lane indicating a total RNA as a loading control.
4.5. Insect bioassay

An insect bioassay was conducted to determine if the levels of dsRNA expressed in the transgenic plant would be sufficient to knock down the gene expression in *H. armigera*. The selected T₀ transgenic plants of both *HaSP* and *HaJHAMT* were primarily examined for insect bioassay.

4.5.1. *In vivo* bioassay using dsRNA expressing *HaSP* tomato plants

4.5.1.1. Feeding of transgenic leaf expressed *HaSP* dsRNA induced Mortality in *H. armigera*

The compound leaf detachment bioassay was performed using the transgenic tomato plant producing dsRNA to examine if the leaf ingested by *H. armigera* larvae could lead to digestive interference by silencing the SP transcript in the midgut, as *SP* is a digestive enzyme in the midgut. Two day old *H. armigera* larvae reared on semi synthetic medium were released on the plates of different lines of transgenic tomato leaf expressing dsRNA of *HaSP*. For this experiment, three (SP3, SP9 and SP15) independent *HaSP* transgenic plants were used. The first day, uniform feeding was observed in all the control and treatment plates (Fig. 4.39). For this, extent of feeding of leaf was visually assessed. The larvae were less fed on the transgenic plant leaf as compared to wild-type (W) and vector control (VC) plants, which were heavily fed. It was observed that, mortality appeared on 3ʳᵈ day and on 4ᵗʰ day maximum mortality was observed, on an average mortality of 20-38% was observed in SP3, SP9 and SP15 and it was 10% in both the controls (Fig. 4.40). It was also observed that there was a reduction in the size of the larvae in the dsRNA expressing *HaSP* leaf feeding compared to vector control and wild-type. The results of this experiment demonstrated an interference of dsRNA on digestive system of the larvae there by affecting the insect growth and development negatively.
Figure 4.39: Compound leaf detachment bioassay, the transgenic leaf expressed HaSP dsRNA ingested *H. armigera* larvae showed reduced leaf damage as compared wild-type and vector control.

Figure 4.40: Mortality of the *H. armigera* was observed after ingestion of the transgenic leaf expressing dsRNA of the insect transcript *SP*. Error bars represents SEM.
4.5.1.2. Assessment of gene silencing of SP in H. armigera by qRT-PCR

To assess the silencing of SP gene in H. armigera qRT-PCR was performed on six days of bioassay to quantify gene silencing signals; qRT-PCR results showed that there was depletion in the transcript of SP. A significant down regulation of the SP transcript to the extent of 98.42% was recorded as compared to leaf fed on wild type and vector control. Between the controls there was no variation in the SP transcript level (Fig. 4.41). The silencing of the SP transcript in H. armigera was achieved using the dsRNA expressing HaSP transgenic tomato plant.

![Graph showing relative expression of SP gene](image)

**Figure 4.41:** qRT-PCR analysis for the down regulation of SP transcript in H. armigera larvae fed with HaSP transgenic and non-transgenic leaf on the six day of bioassay. The values represented are means of relative quantity of SP transcript and its standard error at p>0.01.

4.5.2. **In vivo** bioassay using dsRNA expressing HaJHAMT tomato plants

A preliminary bioassay was conducted to select the T1 generation HaJHAMT tomato for advancing the generation. Second instar H. armigera larvae were fed on the detached compound leaves of two selected lines, JH2 and JH40 vector control and wild type leaves served as controls. Down regulation of the JHAMT gene in these larvae was assessed using qRT-PCR.
4.5.2.1 Feeding of dsRNA expressed *HaJHAMT* transgenic leaf reduced body weight in *H. armigera*

In the compound leaf-let feeding bioassay using 2\textsuperscript{nd} instar larvae, initially for the first two days no visual differences were observed in the larval feeding and growth rate. However, from 3\textsuperscript{rd} day of the bioassay a reduction in larval feeding was observed in the larvae fed on transgenic leaves compared to the control leaves (Fig. 4.42\textit{b}) and consequently the larval growth was affected in the larvae fed on the transgenic leaves, an average daily larval weight gain of less than 50\% at 22.39 mg in JH2-A2 fed larvae compared to 45.06 mg and 52.86 mg in wild-type and vector control leaf fed larvae respectively (Fig. 4.43 and Table 4.3). The larvae fed on wild-type and vector control leaves showed normal growth (larval size) and development and appeared healthy compared to those fed on JH2A-2 and JH2B-6 transgenic leaves (Fig. 4.42\textit{a}). The larval movement in transgenic leaves was lower compared to those fed on control leaves.

Visual observations were resorted to minimize the larval handling in young larvae and larval weight was recorded from 7\textsuperscript{th} to 12\textsuperscript{th} day of the bioassay. A severe reduction in larval weight was observed in transgenic leaf fed larvae treatment plate as compared to controls. The average weight gain recorded in JH2A-2 and JH2B-6 fed larvae were 135.5 mg and 262.7 mg and for vector control and wild-type were 354 mg and 359 mg respectively, statistically the treatments were significant over both controls (p>0.05) (Fig. 4.44 and Table 4.3). The reduction in the weight of the larvae in JH2A-2 leaf fed was 62.23\% and in JH2B-6 leaf fed larvae was 26.85\% respectively, compared to the wild-type control leaf fed larvae.

4.5.2.2 Feeding of transgenic leaf expressed *HaJHAMT* dsRNA induced mortality in *H. armigera*

The feeding of transgenic leaf induced mortality in *H. armigera* larvae due to the silencing of the *JHAMT* gene. The mortality began before the entry into pupal stage in transgenic fed larvae but were absent in controls. The larval and prepupal mortality rates were significantly higher in the larvae fed on leaves of transgenic tomato, a larval mortality of 68.75\% and 41.18\% in JH2A-2 and JH2B-6 leaf fed
larvae respectively, and the remaining larvae underwent incomplete pupation and died subsequently in JH2A-2, while only 35.29% of the pupae formed adults in JH2B-6 compared to the 100% eclosion in both the controls (Table 4.3).

Figure 4.42: Insect bioassay with dsRNA expressing HaJHAMT tomato plants (a) size reduction in the larvae fed on T$_2$ transgenic plants (b) Larval bioassay with detached-leaf. VC, Vector control and WT, Wild-type tomato plants
Figure 4.43: Growth of *H. armigera* larvae. Average weight gain of the larvae from 7th to 12th day.

4.5.2.3. Feeding of dsRNA expressed *HaJHAMT* transgenic leaf reduced pupal weight in *H. armigera*

The transgenic leaf fed larvae showed reduction in the pupal weight and in size compared to controls. The pupal weight was significantly lower and smaller pupae were observed in

Figure 4.44: Average weight of larvae and pupae recorded on 12th day and after pupation respectively. The error bars indicate standard error means of all the replicates. VC- Vector control and Wild type tomato plants.
the larvae fed on transgenic leaves compared to the controls. A reduction in pupal weight of 36.9% and 26.8% were observed in pupae derived from the larvae fed on JH2A-2 and JH2B-6 leaves respectively (Fig. 4.44) and were statistically significant (p>0.05). Pupation rate was drastically reduced in the larvae fed on HaJHAMT transgenic leaves, only 31.25% and 58.82% of pupation was observed in the larvae fed on JH2A-2 and JH2B-6 leaves respectively (Table 4.3). The entry of the larvae to metamorphosis was by 3 and 1.5 days in the larvae fed on JH2A-2 and JH2B-6 leaves respectively, compared to the average duration in vector and wild type controls (Table 4.3).

4.5.2.4. Developmental deformities and incomplete metamorphosis in the larvae fed on transgenic leaves

Developmental abnormalities were observed due to incomplete metamorphosis, larval-pupal intermediates were observed only in the larvae fed on the HaJHAMT

Figure 4.45: Phenotypic aberrations in metamorphosis (A) Close-up of the larva fed on JH2A-2 leaves, to the left is the anterior portion of the larvae and to the right is the posterior portion of the larvae (B) Pupal size and developmental abnormalities in larvae fed on JH2A-2 and JH2B-6 leaves.
transgenic leaves, while in control no such abnormalities were noticed (Fig. 4.45B). The larvae attained slowly pupation in JH2A-2 and JH2B-6 fed leaves, and showed difficulty to shed cuticle, and were died. And some went to pupation with very difficult and were smaller in pupal size but most of them failed to form adults in JH2A-2 fed leaves, however very few from JH2B-6 fed larvae were pupated. The close-up of the larva (Fig. 4.45A) depicts the incomplete apolysis due to the inability of the larva to shed the larval cuticle during the larval-pupal molt.

4.5.2.5. Transcript level of JHAMT in H. armigera larvae

Two independent transgenic pGRNAi-HaJHAMT lines JH2 and JH40 were analyzed in preliminary in vivo insect bioassay for their JHAMT levels in H. armigera using qRT-PCR. On 4th day of bioassay, it was observed that the silencing of the JHAMT levels in JH2 derived plants JH2-1 and JH2-5 were 68-70%, similarly in JH40 derived plants JH40-3 and JH40-1 was 40-67% (Fig. 4.46) respectively, and consistent silencing was observed in two plants of the line JH2 plants compared to another line JH40. The line JH2 was advanced to T2 generation for further analysis. In T2 generation HaJHAMT transgenic plants, it was found that on 6th day, the transcript levels of JHAMT were depleted by 90% and 84.3% in JH2A-2 and JH2B-6 leaf (Fig. 4.46).

![Graph](image)

**Figure 4.46:** Assessment of expression levels in *H. armigera* (a) Transcript levels of JHAMT in *H. armigera* larvae fed on T1 and T2 pGRNAi-HaJHAMT tomato plants.
Table 4.3: Insect bioassay with T2 HaJHAMT plant, depicting overall effect on growth and development of H. armigera. Values with the same letter are not significantly different at the p>0.05 level.

<table>
<thead>
<tr>
<th></th>
<th>Average larval weight (mg)</th>
<th>Average daily weight gained(mg)</th>
<th>Mortality (%)</th>
<th>Pupation (%)</th>
<th>Pupal mortality (%)</th>
<th>Mean weight of pupae</th>
<th>Pupation period</th>
<th>Adult formed %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>359.0 a</td>
<td>45.06 a</td>
<td>0</td>
<td>100.00 a</td>
<td>0</td>
<td>222.0 a</td>
<td>17-20</td>
<td>100.00</td>
</tr>
<tr>
<td>VC</td>
<td>354.0 a</td>
<td>52.86 a</td>
<td>0</td>
<td>100.00 a</td>
<td>0</td>
<td>222.4 a</td>
<td>15-20</td>
<td>100.00</td>
</tr>
<tr>
<td>JH2A-2</td>
<td>135.5 b</td>
<td>22.39 b</td>
<td>68.75 a</td>
<td>31.25 b</td>
<td>100</td>
<td>143.2 b</td>
<td>18-24</td>
<td>0.00</td>
</tr>
<tr>
<td>JH2B-6</td>
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<td>44.50 a</td>
<td>41.18 b</td>
<td>58.82 c</td>
<td>40</td>
<td>164.2 b</td>
<td>18-21</td>
<td>35.29</td>
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</tbody>
</table>