Appendix
Appendix 8.1 Preparation of bacterial culture media

Yeast extract monnitol medium (YEMA)

All the below ingredients except agar were added and dissolved in 950 mL of double distilled water double distilled water, finally the pH of the medium was adjusted to 7.0 and volume was brought up to one liter with double distilled water. The medium was stream sterilized and stored at +4°C.

<table>
<thead>
<tr>
<th>YEMA media components</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td>10.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.4</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1</td>
</tr>
<tr>
<td>MgSO4.7H2O</td>
<td>0.2</td>
</tr>
<tr>
<td>K H2PO4</td>
<td>0.5</td>
</tr>
<tr>
<td>Agar</td>
<td>15.0</td>
</tr>
</tbody>
</table>

YEM-Agar plates

It was prepared as described above but 15g/L of agar was added prior to autoclaving. After autoclaving, it was cooled to approximately 55°C and desired antibiotics were added and poured into petri dishes. After solidification, the petridishes were invert stored at +4°C.

Luria Bertani (LB) medium

One liter of the LB medium was prepared by dissolving below mentioned ingredients in 950 mL of double distilled water and pH of the medium was adjusted to 7.0 using 1N NaOH, volume was brought up to 1 liter using double distilled water. The solution was autoclaved and stored at +4°C.
<table>
<thead>
<tr>
<th>LB media components</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>10.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>10.0</td>
</tr>
</tbody>
</table>

**LB agar-plates**

It was prepared as described above but 15g/L of agar was added prior to autoclaving. After autoclaving, it was cooled to approximately 55°C and desired antibiotics were added and poured into petri dishes. After solidification, the petridishes were invert stored at +4°C.

**SOB medium (Hanahans medium)**

All the ingredients listed below were dissolved in 950 mL of double distilled water and 10 mL of 250 mM solution of KCl was added (KCl was prepared separately by dissolving 1.6 g in 100 mL of double distilled water). The pH of the medium was adjusted to 7.0 and after adjusting the medium volume to 995 mL with double distilled water it was autoclaved. After autoclave 5 mL of 2 M MgCl₂ was added (it was prepared by dissolving the 19 g of MgCl₂ in 100 mL of double distilled water).

<table>
<thead>
<tr>
<th>SOB media components</th>
<th>g/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>20.0</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**1M Glucose**

One molar glucose was prepared by adding 18 g of glucose to 100 mL of double distilled water. The prepared solution was membrane filter sterilized using 0.22μ filter and stored at +4°C.

**SOC medium**

It was prepared by adding 20 mL of 1 M glucose (membrane filter sterilized) to autoclaved SOB medium and was stored at +4°C.
Appendix 8.2 Preparation of selection reagents and antibiotics

**Ampicillin stock solution (50 μg/mL)**

A quantity of 2.5 g of ampicillin sodium salt was dissolved in 50 mL of double distilled water, filter sterilized, aliquoted in 1.5 mL tubes and stored at -20°C.

**Kanamycin (100 mg/mL)**

One gm of Kanamycin was dissolved in 10 mL of sterilized double distilled H₂O, filter sterilized with 0.22 μm membrane filter, dispensed in sterilized 1.5 mL tubes and stored at -20°C.

**Augmentin™ (300 mg/mL)**

A quantity of 1.2 g of Augmentin was dissolved in 4 mL of double distilled H₂O filter sterilized with 0.22 μm membrane filter, dispensed in sterilized 1.5 mL tubes and stored at -20°C.

**Tetracycline (5mg/mL)**

Tetracycline was prepared by dissolving 25mg in little quantity of alcohol then volume was made up to 2.5 mL using autoclaved double distilled water. The prepared antibiotic was membrane filter sterilized, then aliquoted into autoclaved 1.5 mL microcentrifuge tubes covered with aluminum foil to prevent light reactions.

**X-Gal stock solution (20 mg/mL)**

X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) of 200 mg was dissolved in 10 mL N, N-dimethylformamide and stored at -20°C in the dark and 40 μL of X-Gal was used for spreading on Luria Bertani (LB) agar plate.

**IPTG stock solution (100 mM)**

IPTG (isopropyl-β-D-thiogalactopyranoside) of 1.2 g was dissolved in 50 mL double distilled water, filter-sterilized and aliquots were stored at 4°C. 40 μL of the solution was used per plate.
Appendix 8.3 Preparation of reagents for electrophoresis and reagents for plasmid isolation

1M Tris base

It was prepared by dissolving 60.57 g Tris (hydroxymethyl) aminomethane (Sigma- Aldrich, USA) in 400 mL of double distilled water and the pH was adjusted to 7.5 using HCl and the final volume was made up to 500 mL using double distilled water. The prepared solution was autoclaved and stored at +4°C.

EDTA 0.5M

It was prepared by dissolving 186.1g of diaminooethane tetraacetic acid (Sigma- Aldrich, USA) in 400 mL of double distilled water by adjusting the pH to 8.0 using 5M NaOH. The prepared solution was autoclaved and stored at +4°C.

NaCl 5M

It was prepared by dissolving 292 g of NaCl in 800 ml of double distilled water and the volume was made up to one liter with deionized H2O. The prepared solution was autoclaved and stored at +4°C.

5M NaOH

100 g of NaOH pellets were dissolved in a clean one litre beaker on ice bucket and the solution was autoclaved in amber bottle. The solution was stored at room temperature.

SDS 10%

A quantity of 10 g sodium lauryl sulfate was dissolved in 100 mL of autoclaved double distilled water, filter sterilized and stored at room temperature.

Potassium Acetate 5M

It was prepared by dissolving 49.07 g of potassium acetate in 70 ml of double distilled water then final volume was made to100 ml using double distilled water. The prepared solution was autoclaved and stored at +4°C.
Alkaline Lysis Solution I

Solution I was prepared by adding 5 mL of 1M of glucose, 10 mL of 1M Tris (pH 8.0) and 1 mL of 0.5M EDTA in the final molar concentration of 50mM, 25mM and 10mM respectively. The prepared solution was adjusted to 100mL, autoclaved and stored at +4°C.

Alkaline Lysis Solution II

Solution II was freshly prepared during the plasmid isolation, 0.4 mL of 5N NaOH and 1 mL of 10% SDS were added in the final concentration of 0.2N and 1% respectively, to a clean 30 mL vial and then the volume of the solution was made up to 10 mL using autoclaved double distilled water. The prepared solution was stored at room temperature.

Alkaline Lysis Solution III

Solution III was prepared by adding 60 mL of 5M potassium acetate and 11.5 mL of glacial acetic acid and the solution volume was made up to 100 mL using double distilled water. The solution was autoclaved and stored at +4°C.

$T_{10}E_1$ buffer

$T_{10}E_1$ buffer was prepared by adding 10 ml of 1 M Tris buffer (pH 7.5) and 2 mL of 0.5M EDTA (pH 8.0) and the volume was adjusted one liter with double distilled water. The prepared solution was autoclaved and stores at +4°C.

DNA Extraction buffer

It was prepared by the addition of below mentioned components, once after complete dissolving of the CTAB at 65°C, which was used as an extraction buffer for DNA isolation.
Components of DNA extraction buffer

<table>
<thead>
<tr>
<th></th>
<th>Final concentration</th>
<th>For 50 mL</th>
<th>For 100 mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular biology grade H₂O</td>
<td>100.0 mM</td>
<td>29 mL</td>
<td>58 mL</td>
</tr>
<tr>
<td>Tris 1 M</td>
<td>1.5 M</td>
<td>5 mL</td>
<td>10 mL</td>
</tr>
<tr>
<td>NaCl 5 M</td>
<td>10.0 mM</td>
<td>15 mL</td>
<td>30 mL</td>
</tr>
<tr>
<td>EDTA 0.5 M</td>
<td>2.0%</td>
<td>1 mL</td>
<td>2 mL</td>
</tr>
<tr>
<td>B-Mercaptoethanol</td>
<td>2.0%</td>
<td>1 mL</td>
<td>2 mL</td>
</tr>
<tr>
<td>CTAB</td>
<td></td>
<td>1 g</td>
<td>2 g</td>
</tr>
</tbody>
</table>

**Agarose gel preparation**

Depending on the size and molecular weight of DNA, the percentage of agarose gel was prepared, the below composition of agarose and 50X TAE was used for preparation of 120 mL gel.

<table>
<thead>
<tr>
<th>Agarose</th>
<th>For 120 mL gel</th>
<th>50X TAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8%</td>
<td>960 mg</td>
<td>2.4 mL</td>
</tr>
<tr>
<td>1.0%</td>
<td>1200 mg</td>
<td>2.4 mL</td>
</tr>
<tr>
<td>1.2%</td>
<td>1400 mg</td>
<td>2.4 mL</td>
</tr>
</tbody>
</table>

**TAE electrophoresis buffer 50X**

It was prepared by dissolving 242 g of tris base in 800 mL of double distilled water and to this 100 mL of 0.5 M EDTA and 57.1 mL of glacial acetic acid was added and the pH was adjusted to 8.0.

**Loading dye 6X**

6X loading buffer was prepared in 50 mL beaker by adding the 3.6 mL of 60 mM EDTA, 0.3 mL of 10 mM tris HCl (pH 7.6), 45 mg orange G dye, 9 mg of xylene cynol and 18 mL of 60 % of glycerol, and finally the volume was adjusted to
30 mL with 7.1 mL of sterilized double distilled water. The prepared 6X loading dye was aliquoted into 1.5 mL microcentrifuge tubes and stored at -20°C.

**Appendix 8.4 Preparation of transformation reagents**

**Transformation buffer**

Inoue transformation buffer (TB) was prepared by dissolving 10.8 g of MnCl₂4H₂O, 2.20 g of CaCl₂2H₂O and 18.65 g of KCl at the final concentration of 55 mM, 15 mM and 250 mM respectively in 800 mL of double distilled water and then 20 mL of 5 M PIPES (pH 6.7). The prepared TB buffer was membrane filter sterilized and stored at -20°C.

**0.5 M PIPES (pH 6.7)**

 Piperazine-1,2-bis[2-ethanesulfonic acid] (PIVES) was prepared by dissolving 15.1 g PIPES in 80 mL of double distilled water. Then the pH of the solution was adjusted to 6.7 using 5M KOH. The final solution volume was adjusted to 100 mL using double distilled water. The solution was sterilized using membrane filter and was stored at -20°C.

**0.1M HEPES buffer (pH 7.5)**

HEPES buffer was prepared by dissolving 2.38 g in 80 mL of water and the pH of the solution was adjusted to 7.5 using NaOH. The prepared solution was membrane filter sterilized and stored at -20°C.

**10% Glycerol**

Glycerol of 10 mL was dissolved in 90 mL of double distilled water and autoclaved and stored at +4°C.
Appendix 8.5 Preparation of tissue culture media

Muarshige and Skoog medium stock components

<table>
<thead>
<tr>
<th>Micronutrients:</th>
<th>1X (mg/L)</th>
<th>400X (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Components</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>0.025</td>
<td>10</td>
</tr>
<tr>
<td>CuSO₄.5H₂O</td>
<td>0.025</td>
<td>10</td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>6.2</td>
<td>2480</td>
</tr>
<tr>
<td>KI</td>
<td>0.83</td>
<td>332</td>
</tr>
<tr>
<td>Na₂MoO₄.2H₂O</td>
<td>0.25</td>
<td>100</td>
</tr>
<tr>
<td>MnSO₄.H₂O</td>
<td>16.9</td>
<td>6760</td>
</tr>
<tr>
<td>ZnSO₄.7H₂O</td>
<td>8.6</td>
<td>3440</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Macronutrients</th>
<th>1X (mg/L)</th>
<th>40X (gm/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Components</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>440</td>
<td>17.6</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>170</td>
<td>6.8</td>
</tr>
<tr>
<td>KNO₃</td>
<td>1900</td>
<td>76.0</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>370</td>
<td>14.8</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>1650</td>
<td>66.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Iron source</th>
<th>1X (mg/L)</th>
<th>200X (gm/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Components</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FeNaEDTA</td>
<td>36.7</td>
<td>7.34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>1X (mg/L)</th>
<th>400X (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Components</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>2.0</td>
<td>800</td>
</tr>
<tr>
<td>Myo-inositol</td>
<td>100.0</td>
<td>40000</td>
</tr>
<tr>
<td>Nicotinic HCl</td>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>Pyridoxine HCl</td>
<td>0.5</td>
<td>20</td>
</tr>
<tr>
<td>Thiamine HCl</td>
<td>1.0</td>
<td>400</td>
</tr>
</tbody>
</table>

MS medium components for Seed germination

Half strength (0.5X) MS medium components (given below) except industrial agar were added to a clean beaker containing 800 mL of double distilled water. After adjusting the medium pH to 5.8, industrial agar was added and then melted in micro oven and dispensed into wide mouth bottles and stem sterilized.

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Appendix 8.6 Preparation of Southern and Northern blotting reagents

The following reagents were prepared according to the standard procedure described in the DIG manual entitled ‘procedures for nonradioactive labeling and detection’.

**Depurination solution**

HCl of concentration 0.25M was prepared by adding 10.4 mL of 37% HCL into the glass beaker contained 489.6 mL of double distilled water. The prepared solution was autoclaved and stored at RT.

**Denaturation solution**

The denaturation solution was prepared by adding 50 mL of 5M NaCl and 150 mL of 5M NaOH in the final concentration of 0.5 M and 1.5 M respectively, the volume of the solution was adjusted with autoclaved double distilled water to 500 mL. The prepared solution was autoclaved and stored at RT.

**Neutralization solution**

Neutralization solution was prepared by adding 250 mL of 0.5M Tris-HCl and 150 ml of 5M NaCl at the final concentration of 0.5M and 1.5M respectively, after adjusting the pH of the solution to 7.5, the volume was made to 500 mL using autoclaved double distilled water.

---

### MS Medium salts

<table>
<thead>
<tr>
<th></th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macro salts (40X)</td>
<td>12.50 mL</td>
</tr>
<tr>
<td>Micro salts (400X)</td>
<td>1.25 mL</td>
</tr>
<tr>
<td>FeNaEDTA (200X)</td>
<td>2.50 mL</td>
</tr>
<tr>
<td>Sucrose</td>
<td>7.50 gm.</td>
</tr>
<tr>
<td>Industrial agar</td>
<td>7.00 gm.</td>
</tr>
</tbody>
</table>
20X Saline sodium citrate (SSC)

The stock solution of 20X SSC was prepared by adding 175.30 g of 3M NaCl and 88.2 g of 0.3M sodium citrate in 800 mL of double distilled water. The pH of the solution was adjusted to 7.0 then was autoclaved and stored at room temperature.

Hybridization solution

DIG easy Hybridization granules (Roche diagnostics, Germany) were prepared by directly adding 64 mL of autoclaved double distilled water to the plastic bottle. The solution was dissolved on water bath at 37°C and stored at -20°C.

High stringency wash buffer

High stringency buffer was prepared by adding 12.5 mL of 20X SSC at the final concentration of 0.5X and 500 mg of SDS (0.1%) 487.5 mL of autoclaved double distilled water.

Low stringency wash buffer

Low stringency buffer was prepared by adding 50 mL of 20X SSC at the final concentration of 2X and 500 mg of SDS (0.1%) in 450 mL of autoclaved double distilled water.

Maleic acid buffer

Washing buffer was prepared by adding 11.6 g of 0.1 M maleic acid and 8.77 g of 0.15M NaCl in 800 mL of double distilled water. The pH of the solution was adjusted slowly by adding to NaOH pellets to attain pH 7.5 and then the solution volume was made to 1000 mL and was autoclaved and stored at room temperature.

Washing buffer

Washing buffer was prepared by adding 1.5 mL of 0.3% tween 20 to the 500 mL of maleic acid buffer. The solution was stored at RT.
Blocking stock solution 10X

10X Blocking reagent (Roche diagnostics, Germany) was prepared by adding 10 g in 100 mL of maleic acid buffer in water bath at 65°C. The solution was autoclaved after complete dissolving of the blocking reagent and stored at -20°C.

Blocking solution 1X

1X blocking solution was prepared freshly by mixing 10 mL of the 10X blocking solution with 90 ml maleic acid buffer.

Detection buffer

Detection buffer was prepared by adding 10 mL of 1M Tris and 2 mL of 5M NaCl to 60 mL of autoclaved double distilled water, at the final concentration of 0.1M and 0.1M respectively. The pH of the detection buffer was adjusted to 9.5 and then volume was made to 100 mL with water. The solution was autoclaved and stored at +4°C.

MOPS buffer 10X

MOPS 10 X buffer was prepared by dissolving 41.92 g of 200 mM MOPS (3-Morpholinopropanesulfonic acid), 4.1 g of 50 mM sodium acetate and 40 mL of 0.5M EDTA at the final concentration of 20 mM in a 900 mL of DEPC treated autoclaved double distilled water. After adjusting the pH of the solution to 7.0, it was membrane filter sterilized and stored in amber bottle wrapped with aluminum foil at +4°C.

Loading buffer for RNA

It was prepared by adding 250 μL of 100% formamide,83 μL of 37% formaldehyde.
Use of proximal hypocotyl segment for high-throughput transgenic development of tomato


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Abstract

Achieving high-throughput and efficient regeneration are the top priorities of any Agrobacterium-mediated plant transformation experiments to develop large numbers of in vitro transsistants. The type of explant plays a critical role in shoot regeneration efficiency. In the present investigation, an attempt was made to study the effect of various segments of hypocotyl and abaxial/adaxial orientation of cotyledon explants on regeneration efficiency in tomato. A plant transformation vector, pGRNAi-harboring dsRNA expressing constructs targeted to two genes of Helicoverpa armigera, serine protease and chymotrypsin independently were used to transform tomato. Of the three segments of hypocotyls obtained from 12 day old seedlings, the proximal (closest to shoot apex) segment had yielded highest regeneration (28.65%) compared to the middle (11.86%) and the distal segments (11.20%). In cotyledon explants, those incubated with their abaxial surface in contact with media exhibited highest regeneration (20.83%) compared to adaxially placed cotyledon explants. However, of the two-explant types, hypocotyls had higher regeneration compared to cotyledons. The molecular characterization of putative transformants through PCR and Southern blot analysis revealed the presence of the transgene. Thus, these results will aid in obtaining high-throughput regeneration in transformation of tomato in particular and other crops in general.

Highlights:

• Best explant for tomato transformation is the proximal portion of hypocotyl where highest regeneration efficiency was obtained.
• Abaxial incubation of cotyledon yielded higher regeneration than adaxial.

Keywords: Cotyledon, Hypocotyl, PCR, regeneration efficiency, Southern blotting

Tomato (Solanum lycopersicum L.) is a globally cultivated vegetable crop ranking second after potato and it belongs to the family Solanaceae. due to its high nutritional value, it is extensively consumed fresh, cooked as well as in processed form. It is rich in antioxidants, carotenoids, lycopene and other nutrients. Every year around 150 million metric tons of tomatoes were produced globally (FAO 2012). In India, its productivity was 19.9 metric tons per hectare during 2010. With the advent of the next generation sequencing, massive sequence information is made available in a number of crops, thus necessitating the
use of high-throughput functional validation tools such as RNA interference, which in turn require high-throughput plant transformation protocols. Plant transformation is also an important tool in genetic enhancement of crops including the traits such as tolerance to biotic and abiotic stresses and improved post-harvest as well as agronomic traits.

Employing the recent advancements in the field of molecular biology such as RNA interference and site-directed genome editing tools such as ZFNs (zinc-finger nucleases) and CRISPR/cas9 (clustered regularly interspersed short palindromic repeat/Crispr-associated protein 9) and various functional genes have been elucidated and metabolic pathways have been traced by gene silencing or by the down regulation of target genes. Utilizing Agrobacterium mediated gene transformation and RNAi approach, today a number of gene functions in various crop plants has been efficiently characterized (Xionget et al., 2013; Zhu et al., 2012). Furthermore, traits of interest like herbicide tolerance, nutritional enhancement and bio-fortification by the accumulation of folic acid, anthocyanin enhancement (Butelliet al., 2008; Maligeppagolet al., 2013), delayed ripening of fruit (Gupta et al., 2013; Klee et al., 1991), drought, salinity and heat tolerance traits (Shahaet al., 2013; Maheshet al., 2013) have been engineered.

Among the Solanaceae crop tomato has been widely used in the Agrobacterium mediated transformation experiments for the trait development due to the rationale that it has small genome size, amenability for genetic manipulation, availability of the annotated genome sequence, (Sato et al., 2012) etc., because of these facts today tomato has been considered as a model plant next to tobacco. As a result, it has been used extensively to study the fruit development and ripening, furthermore it is used to help unraveling the mysteries in functional genomics, proteomics, and metabolomics. After McCormick's (1986) first report on Agrobacterium mediated tomato transformation, today a number of papers have been published, describing the regeneration and transformation efficiency, with various parameters, like, type of the hormone concentration, selection medium, cocultivation method, time duration of cocultivation, genotype, age and type of the explants etc., (Devi et al., 2013; Sharma et al., 2012). Among these parameters, explant plays an important role in determining the regeneration efficiency, choice of the explant and the orientation of incubation in the culture medium, i.e., polarity of hypocotyls and orientation of cotyledon determines the success of regeneration (Sharma et al., 2009; Bhatia et al., 2005; George, 1993). So far, explants such as, leaf, root, hypocotyl, cotyledon, and petiole have been used for tomato regeneration, (Sharma and Srivastava, 2014) and different regeneration responses were obtained. However, for the Agrobacterium mediated transformation in tomato both hypocotyl and cotyledon have been widely used. One of the bottlenecks in the Agrobacterium mediated tomato regeneration is the reproducibility of the protocols among the various cultivars or genotypes used for transformation (Sharma et al., 2009). Hence, it is required to optimize each genotype before it is being used for Agrobacterium mediated tomato transformation for the trait development. In the present study, a good general tomato combiner Arkavikas (Bhatt et al., 2001) was used for transformation. Very few reports are available on tomato cultivar Arkavikas for shoot regeneration and transgenic development (Manamohanan et al., 2011; Shivakumaret al., 2007; Sharma et al., 2009) and to provide the maximum information to utilize this cultivar for the transgenic development an effort has been done. In our previous report, we optimized the protocol to obtain high shoot regeneration percentage using pCAMBIA2301 vector carrying a dreb1A gene with hypocotyl as explant (Manamohanan et al., 2011). In this report it was extended to assess the distinct shoot regeneration efficiency among the hypocotyl segments and also examined the influence of the cotyledon orientation on shoot regeneration by employing ammodified in-house built binary vector pGRNAi (Manamohanan et al., 2013) harboring the HaSP and HaCHY gene.
Fig. 1. Preparation of the explants. The hypocotyl was dissected into three segments and cotyledon orientation.

Fig. 2. 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.
Materials and methods

Preparation of Tissue culture medium

For the Agrobacterium mediated transformation of tomato, the basal medium of Murashige and Skoog (1962) was used. Different types of hormone combination for the seed germination, shoot induction, shoot elongation, and root induction were mentioned in the table 1. All the MS medium components were adjusted to pH 5.8 prior to the addition of 0.3% plant tissue-culture grade agar-agar (Sigma, USA). After that all the components were autoclaved at 121°C for 20 min. The growth regulators 6-benzylaminopurine (BAP) and Indole-3-acetic acid (IAA) were obtained from sigma, 1 mg/ml stock concentration of growth hormones were prepared using a small amount INaOH.

The antibiotics stock kanamycin (100mg/ml) and augmentin (300mg/ml) were prepared in Milli Q water and were stored in -20°C. Antibiotics and growth hormones were added to the autoclaved MS medium after it cooled to ~50°C.

Seed germination

Tomato cv. Arka Vikas seeds were obtained from the Division of Vegetable Crops, Indian Institute of Horticultural Research (IIHR), Bengaluru. Primarily the seeds were surface sterilized by immersing in 70% v/v ethanol for seven min, immediately seeds were rinsed three times with autoclaved double distilled water. The surface sterilized seeds were immersed in fresh 4% sodium hypochlorite solution for 10 min followed by washing with sterilized water for several times. Seeds were blot dried on sterilized

Fig. 3. Statistical analysis of the shoot regeneration efficiency of the hypocotyl type explants using 't' test. A) 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). B) Influence of cotyledon orientation on the shoot regeneration efficiency. C) The highest regeneration efficiency was observed in abaxial orientation in contrast to adaxial orientation and were statistically significant (p>0.05). D) Regeneration efficiency between hypocotyl and cotyledon. (Values represented in percent mean ± SE).
tissue paper and were sown in seed germination medium (Table 1) in culture bottles. Culture bottles were kept initially for two days in dark at 25±1°C. and later were exposed to photoperiod of 16/8 h in culture racks with an illumination of light intensity 40-60 µmol/sec.

Gene constructs preparation

Two *H.armigerata* target genes serine protease (HaSP, 500 BP) and chymotrypsin (HaCHY, 465 BP) fragments were selected. Both the gene fragments were PCR amplified using primers incorporating the restriction sites BamHIin forward and SalI reverse primers. The RNAi assemblies for individual genes were prepared using pGRNai vector (Manamohan et al., 2013), the gene constructs were prepared with HaSP and HaCHY genes and are designated as pGRNai-HaSP and pGRNai-HaCHY respectively. Both the binary vectors were electroporated into the *Agrobacterium tumefaciens* strains EHA 105 containing pSoup plasmid. The electroporated clones were selected in yeast extract mannitol (YEM) agar medium containing antibiotics, i.e., 10 mg/L rifampicin, 10 mg/L tetracycline and 50 mg/L kanamycin.

*Agrobacterium tumefaciens* strain

*Agrobacterium* strain EHA 105 was used for transformation, the cells were streaked and maintained in YEM medium supplemented with 100 mg/L kanamycin. Single colony from the plate was picked in YEM broth with 100 mg/L kanamycin and incubated at 28°C, once the culture attained the 0.1 OD600 and then it was used for co-cultivation of the explants.

Explant preparation

Tomato seedlings of twelve day old were used for the preparation of explants, the top 5-6 mm highly regenerative, meristematic portion of the hypocotyl was removed and rest was used for the preparation of hypocotyl segments. The hypocotyl was cut and categorized into three segments, proximal, middle, distal and were designated as HYPSeg-I, HYPSeg-II and HYPSeg-III respectively (Fig. 1). All the hypocotyls segments were cut with the size of 0.8 to 1 cm in length and were incubated separately onto the MS medium. For the cotyledon explant preparation, a small portion of cotyledons from both the end was excised and placed on the MS medium in both abaxial and adaxial orientation.

*Agrobacterium* mediated transformation

The explants prepared were transferred to the preculture medium. Hypocotyl segments thus categorized were placed separately on the MS medium, similarly, cotyledons were placed in

Fig. 4 Molecular analysis of the primary putative transgenic plants. A) the PCR confirmation of the npII gene M: Molecular Weigh Marker- DNA Hyper ladder-I (Bioline), sample nos 1, 2, 4, 17 and from 6 to 15 containing PCR product of 1.1 kb indicating the presence of the transgene in these plants, -ve : untransformed tomato plant, +ve : positive control (plasmid containing transgene). Fig 4B. Southern blot analysis of the of the selected putative transgenic plants, Lane no 1, 2, 3, 8 and 11 hybridization signals were observed, -ve ; no hybridization signal in the wild type tomato plant. +ve ; plasmid with insert sample served positive control.
adaxial and abaxial orientation, and the explants were incubated for two days. After that, the explants were cocultivated with Agrobacterium cell suspension for 10 min with 0.1 OD_{600nm}. The explants were blotted dry with sterile tissue towel and were shifted once again to fresh preculture medium for 48 hrs. After that, all the explants were transferred to the plates containing MS medium supplemented with augmentin and incubated for 48 hrs, to eliminate the Agrobacterium. The explants were finally shifted to the antibiotic selection medium contained kanamycin and augmentin. The explants were incubated in the antibiotic selection medium for 3 to 4 weeks, subsequently subcultured to the same fresh medium.

The numbers of shoot regenerates were recorded for all the plates and used for calculating the shoot regeneration efficiency. The shoot regeneration efficiency was calculated by dividing the numbers of shoots regenerated to the total numbers of explant inoculated multiplied by hundred. The healthy, grown shoots were excised and shifted to the elongation medium, then to rooting medium. The well profusely rooted plants were acclimatized and maintained in the biosafety net house.

**DNA Isolation and PCR screening**

The genomic DNA was isolated from the young leaves of putative transgenic plants according to Lodhi et al. (1994). The isolated DNA was quantified using the Nanodrop™. For the PCR reaction 100 ng of template DNA, 10X buffer, 200 μM dNTP’s, 2 units of TaqDNA polymerase (Genei, Bengalure) and 1μM of each primer was used for 25 μl PCR, the primer nptII forward ‘5 GATAACATGAGAAATGAAGTCACG3’ and nos promoter reverse ‘5TCAGAAGAAGTCAAGAAG 3’ was used to amplify the chimeric region of nos promoter and the nptII region. The following reaction conditions were set for amplification, initial denaturation 95°C for 4 min, denaturation 94°C for 30 sec, annealing 54°C for 35 sec, extension at 72°C for 1 min and final extension was performed at 72°C for 10 min, the PCR products were resolved on 1.2% agarose TAE (Tris acetate EDTA) gel and documented.

**Statistical analysis:** For each treatment five replicates with 25 numbers of explant were maintained, the analysis of the data were presented with Student’s t-test utilizing GraphPad prism software (USA).

**Southern blot hybridization analysis**

For the determination of copy number Southern hybridization was performed, a selected number of transgenic plants were analyzed, DNA was isolated utilizing the procedure as described by Allen et al. (2006). About 10 μg of DNA was digested with BamHI restriction enzyme and resolved on 0.7% agarose TAE gel. The separated DNA on the gel was transferred to positively charged nylon membrane using 20X SSC (saline sodium citrate). nptII probe of size 1.1 kb was labeled with Digoxigenin-11-dUTP alkali-labilereagent (Roche diagnostics, Germany) was used. The initial steps of Southern hybridization were followed from depurination to UV crosslinking according to the protocol described in (Sambrook and Russell, 2001) and stringency wash and detection steps were followed according to the manufacturer's instructions. For the detection of the hybridization signals CDP-Star and chemiluminescence detector was employed.

**Results and discussions**

In the present investigation, Agrobacterium mediated transformation of tomato cultivar Arkavikasaw used to determine the morphogenetic capability of hypocotyl segments. The shoot regeneration efficiency was determined and optimized for two widely utilized explants, hypocotyl and cotyledon.

**The regeneration response of hypocotyl segments**

In our previous experiment, we have observed that, use of hypocotyl explants resulted in higher regeneration rates than the cotyledons (Manamohan et al., 2011). In the present investigation, Agrobacterium mediated transformation was carried to determine the morphogenetic potential among the hypocotyl segments. The hypocotyl explants were prepared by dissecting it transversely into three segments (Fig.1) 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 (Fig. 2A).

The regeneration percentage of HYP-seg-I obtained in both the construct was 28.65% and 23.23%. Similarly, in HYP-seg II transformed had 11.86% & 10.79% and for HYP-seg III was 11.20% and 11.61% respectively, (Fig. 3 A&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-seg III, which did not exhibit significant variations in regeneration response in independent experimentation. However, maximum rate of shoot regeneration was obtained in the Hyp-seg III both the vector transformed.

The response of hypocotyl in the induction medium was observed in all the hypocotyl segment types regeneration response by forming calli was observed within 10 to 12 days and the appearance of shoot bud started in 14 to 24 days. In our experiment, we could obtain the highest regeneration percentage in hypocotyls, HYP-Seg-I had responded very well and yielded highest regeneration percentage in contrast to the other two hypocotyl segments. This may be due to the influence of the endogenous content and the polar transport of the phytohormone cytokinins and auxins and the polarity of the explant. The fate of explant organogenesis in vitro is decided by the amount of auxin to cytokinin ratio or balance between the endogenous hormone concentration of the explant to the external supplemented hormones, variation in the endogenous hormone concentration in the explant yields a different regeneration response, i.e., it leads to a callus or shoot or root organogenesis. Perez-jimenez et al. (2014) reported the changes in the endogenous hormone concentration such as zeatin (Z), zeatinriboside (ZR), indole-3-acetic acid (IAA), abscisic acid (ABA), ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), salicylic acid (SA), and jasmonic acid (JA) when the explant was cultured with the external supply of growth hormone in the media. However, in the present study, we did not quantify the hormone composition of the both hypocotyl segments and cotyledon explants, possibly the quantification of the endogenous hormone concentration would give answer to the difference in the regeneration efficiency between the segments and explant types. Similar type of observation were documented in hypocotyls segments of Capsicum, where the proximal dissected part produced shoot buds, central part produced roots and distal parts produced more callus (Manamohan and Mythali, 2011). However, in our experiment on tomato, all the parts of the hypocotyl segments produced the shoots buds. The endogenous presence of the cytokinin in the hypocotyl varies with type of explant and with plant species (Parez-jimenez et al., 2014; Coenen and Lomax, 1998). The changes in the expression level of genes encoding for hormone receptor also contributes the difference in the shoot regeneration efficiency (Wang et al., 2014; Motte et al., 2013; Close et al., 1989). The fate of the cell to undergo shoot or root organogenesis is controlled by expression of Regeneration1 (Rg1) as characterized in tomato cultivar microtom, Rg1 increases the capacity of explant to form shoot or root in the medium (Lombardi-Crestena et al., 2012). The dissected segments of hypocotyls of tomato in the MS medium had showed differential morphogenetic capability, the proximal segment had more morphogenetic ability to form shoot buds than other progressively distant dissected segments, as a result, proximal segment had highest regeneration percentage than others. A number of authors reported the highest regeneration using hypocotyl in tomato (Shivankalyan et al., 2014; Yasmin, 2009; Gubieset et al., 2003), however, this experiment further refined the use of proximal portion of hypocotyl segment to obtain the best regeneration response. To our knowledge, this is the first report on the use of different hypocotyl segments to obtain higher regeneration and the superiority of HYP-seg-I for highest shoot regeneration in the Agrobacterium mediated tomato transformation.
Regeneration response of cotyledon

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 2mg/l and 0.1mg/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.3C), 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 shootbuds started at the cut end of the cotyledons on 10th day and continued for 28 days. The cotyledons that wereplaced adaxially in the antibiotic selection medium were become curved and turned into cup shaped structures (Fig.2C), whereas the abaxially inoculated explants appeared dome shape in the center and some were completely flat in structure (Fig.2B). However, morphogenetic potential of the abaxially placed cotyledon was higher compared to adaxial orientation. A clumps of multiple shoot buds were appeared and were turned intoa rosette form, the well-elongated shoots were excised carefully and were shifted to elongation medium supplemented with GA3 0.1mg/l. The cotyledon explants that were not transformed were turned gradually into brownish yellow in colour and such explants were discarded. Similar type of regeneration response was recorded by Bhatia et al. (2005), they studied the influence of the abaxial and adaxial cotyledon orientation and genotypic dependent regeneration in 10 tomato cultivars, they observed the highest regeneration in abaxial than in adaxial orientation.Rani et al. (2013) also reported the highest regeneration percentage in abaxial orientation in the two tomato cultivar HisarArun and HisarLalit, However, in contrast to our results Duzaymanet et al. (1994)obtained opposite results, but Costa et al. (2000) reported similar type of regeneration response in both the orientation. In Apple, the orientation of the cotyledon explant had positive influence on the regeneration, abaxial orientation of the cotyledon gave a highest regeneration percentage of 39.3 (Zhang et al., 2013). Hence, orientation of incubation plays a key role in regeneration.

The successful recovery of more number of shoots and higher regeneration percentage in abaxially placed cotyledon in the antibiotic selection medium was due to the healthy nature of the explants and the contribution of the external hormonal supply, where as in adaxial orientation, due to the cup shaped structure of cotyledon sheltered the growth of Agrobacterium which inhibited the maximum shoot regeneration, where this Agrobacterium escaped during the blotting step after cocultivation. It was also reasoned that the cut ends of the cotyledon, which were facing upwarfd failed to absorb sufficient nutrients from the medium. The abaxially placed cotyledon explants were well swollen in size in the media compared to adaxially placed cotyledon. A variation in the expression of FILAMENTOUS FLOWER (FIL) and the activity of the microRNA miR165/166 in abaxial side and are specific to the abaxial side (Tameshige et al., 2013). Although cotyledon contributed, more numbers of shoot buds but it was difficult to excise and they could not come out from the medium. All the shoots, which were obtained, were rooted in rooting medium containing 0.5mg/l indole-3-butyric acid (IBA) (Fig.2D) and were shifted to hardening facility, then to a biosafety net house facility for maintenance.

Regeneration of hypocotyl v/s cotyledon:

The regeneration response was compared between hypocotyl and cotyledon. It was recorded highest in hypocotyl 25.76% and lowest in cotyledon 16.31%(Fig.3D) and were statistically significant. So far, a number of explants have been examined and reported diplomatic results on the success of type of explants in highest shoot regeneration, this is probably due to the variation in the genotype. Some opinioned that cotyledon explants are superior (Ajenifujah-Soleboet et al., 2012; Zhang et al., 2012; Bhatia et al., 2005) and some were hypocotyls
in tomato regeneration (Shivankalyaniet al., 2014; Yasmin, 2009), but in our case hypocotyl was superior to cotyledon in terms of shoot regeneration. Although the shoot bud formation of the cotyledons in the antibiotic selection medium was more but most of them were failed to elongate into complete shoots. However, in hypocotyl segments such difficulties were not observed. Similar observations were recorded by Gubišet al. (2003) who obtained highest regeneration in hypocotyl compared to cotyledon as he studied in 13 tomato cultivars, Mathews et al. (2003) have also reported that consistency of high transformation rate using hypocotyl as explants of choice for regeneration using tomato cultivar microtoms and hypocotyls explants offered easiness for manipulation. Similarly, Yasmin, (2009) also reported that his choice of explants in terms of highest regeneration was hypocotyl.

Molecular characterization of the transgenic tomato plants:

All the putative T_0, primary transgenic plants were PCR examined for the presence of insert using nos promoter forward and nptII reverse primers. The transgenic plants amplified the 1.1 kb fragment and confirmed insert gene in the transgenic plant (Fig.4A). Plasmid DNA and DNA from control-untransformed plant were used as a positive and negative control respectively. Selected PCR confirmed plants were assessed for copy number.

The Southern hybridization results indicated the single copy gene integration in the genome(Fig.4B).

Conclusion

The present investigation successfully demonstrated that the proximal segments of hypocotyls as the best explant for the Agrobacterium mediated tomato transformationas they exhibited highest shoot regeneration among the hypocotyl explants as well as the cotyledons. This study also demonstrated the effect of orientation of incubation of cotyledons on the regeneration efficiency, where abaxial incubation yielded higher regeneration than the adaxial incubation. The protocol described here is helps in obtaining rapid and efficient transformation of tomato to achieve higher throughput.

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Original Research Article

RNA interference (RNAi) tool for Insect Pest Management Patenting analysis their impact on incentives and disincentives to undertake research and development

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ABSTRACT

RNA interference is a natural process that affects the level of activity of genes in animals and plants. Flexibility, adaptability, and demonstrated effectiveness of RNA interference technology mean it will have an important place in the future of pest management. As with all patents, there are opportunities, challenges and trade-offs when interim absolute rights are required to spur innovation - RNA interference patents are no different. It has gained importance over the decade which is used in this review as a case study and analysis of the patents granted on RNAi insect pest management was carried out. This study categorizes a number of patents related to RNAi in insect pest management and the development of patentable technologies. The analyses were done using various criteria like patenting trends over the years, assignees playing a major role, comparison of the technology used in different patents and the patenting activity across the insect orders. Despite significant achievements have been made RNAi in insect pest management strategies, the patents and patentable technologies were very limited confined to certain geographical regions. Emphasis must be given to the patents and patentable technologies and this will enable the RNAi technology for the effective control of economically important insect pests. Patent documents related to bacterium RNAi insect pest management contain a trove of technical and commercial information and thus, patent analysis is considered as a useful tool for R&D management and techno economical development. Undeniably, there is broad potential for the application of RNAi technology in pest control, mainly if combined into integrated pest Management strategies.

Introduction

Commercial crops are often the targets of insect attack. Chemical pesticides have been very effective in eradicating pest infestations. However, it is well known that there are several disadvantages to using chemical pesticidal agents. First of all, chemical pesticidal agents are not selective, therefore, on the same time of controlling
target insect, because of the lack of selectivity, they also exert their effects on non-target fauna, often effectively sterilizing a field for a period of time over which the pesticidal agents have been applied. Second, chemical pesticidal agents persist in the environment and generally are slow to be metabolized, if at all. They accumulate in the food chain, and finally in the high predator species, such as human being, where these pesticidal agents act as a mutagens and/or carcinogens, to cause irreversible and deleterious genetic modifications. This kind of accumulation causes to higher predator pest resistance. Thus there has been a long felt need for environmentally friendly methods for controlling or eradicating insect infestation on or in plants, i.e., methods which are selective, environmental inert, non-persistent, and biodegradable, and that fit well into pest resistance management schemes. These environmental safe compositions, including Bacillus thuringiensis (Bt) bacteria and transgenic plants expressing one or more genes encoding insecticidal Bt protein, have been remarkably efficient in controlling insect pest infestation. However, with the increased use of Bt crops, such as corn and cotton, comes the threat that target pests may develop resistance to these toxins. Although Bt-resistant insect populations have not yet been observed in the field, resistant strains have been developed in the laboratory by selection with toxin-impregnated diet (McGaughy, 1985). Thus, beside to work out ways to delay Bt resistance development, it is greatly valuable to find a different mode of action to control pest infestations by single use or combined use with Bt expression strategy (Jian Ye et al. 2012).

RNA Interference (RNAi) provides a potentially powerful tool for controlling gene expression because of its specificity of target selection and remarkably high efficiency in target mRNA suppression. RNAi refers to the process of sequence-specific post-transcriptional gene silencing mediated by short interfering RNAs (siRNAs) (Zamore, et al., 2000; Fire, et al., 1998); Hamilton et al., 1999; Lin et al., 1999). While the mechanics underlying RNAi are not fully characterized, it is thought that the presence of dsRNA in cells triggers RNAi by activating the ribonuclease III enzyme Dicer (Zamore, et al., 2000; Ham mond et al., 2000). Dicer processes the dsRNA into short pieces called short interfering RNAs (siRNAs), which are about 21 to about 23 nucleotides long and comprise about 19 base pair duplexes (Zamore et al., 2000; Elbashir et al., 2001).

Following delivery into cells, the siRNA molecules associate with an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which brings together the antisense strand of the siRNA and the cellular mRNA gene target. RISC cleaves the mRNA, which is then released and degraded. Importantly, RISC is then capable of degrading additional copies of the target mRNA. Although the technique of RNAi has been generally known in the art in plants, C. elegans and mammalian cells for some years, to date little is known about the use of RNAi to down-regulate gene expression in insects. The objective of the present study is to assess the patenting trends of RNA interference (RNAi) as one of the new alternative strategies to reduce damage from insect pests offers new opportunities to increase the production by using more sustainable and ecological friendly agriculture system. This patent analysis intern helps researchers to become familiar with RNAi research, a rapidly growing field where new avenues and techniques are being used to investigate.
insect RNAi mechanisms for the development of pest control in eco-friendly manner for sustainable crop production. This also insights on how to reduce the pesticidal residues in environment.

Methodology

In 2002, RNA interference (RNAi) was proclaimed by *Science* as the “breakthrough technology of the year” and by *Fortune* as a “billion dollar breakthrough.” (Chi-Ham *et al.*, 2010). The recognition of RNAi-mediated gene suppression as an important experimental tool and its potential commercial application is further reflected in the patent landscape related to RNAi-mediated gene suppression, with an increasing number of patent applications seeking exclusive rights to RNAi-based discoveries. To analyze technological innovation, patent analysis was used, because it provides innovative information of individual, organizational, regional and national scientific levels. Patent analysis was also used to map the technological activities at various levels. This article attempts to analyze the patenting activity in the field of RNAi new sound pest management strategy. This report also try to highlight the important technological directions and gaps in our knowledge in order to allow further pursuit of R&D, using data from different databases, namely European Patent and trade mark office database (EPO) (http://www.epo.org/), Google patents (https://www.google.com/?tbm=pts), Indian patent database (IPO) (http://www.ipindia.nic.in/), United States Patent office database (USPTO) (http://patft.uspto.gov/netahtml/PTO/search-bbool.html), World Intellectual Property Organization (WIPO) (http://www.wipo.int/tools/en/gsearch.html). All searches and data have been culled from 1970 to 2013 to cover active patenting authorities throughout the world.

International search for patents on a specific subject used the following key word: RNAi in insect pest management. Searches were made using WIPO, USPTO, EPO, PCI, JPO, KIPO, INPADOC, IPO and all electronic database used the advanced Boolean search on issuing date, country and international classification number and bibliographical references of all the patents. This was done in order to understand the technical approaches taken by different research groups throughout the world. It also provided an insight into emerging technologies and key areas for R&D.

Results and Discussion

Gene silencing has been suggested as one of the new alternatives to reduce damage from insect pests. RNA interference (RNAi) is first described by Fire *et al.*, 1998, and its mechanism lies in that a double-stranded RNA (dsRNA) introduced in an organism has the capacity to silence post-transcriptional genes (Hannon, 2002; Geley and Muller, 2004). RNAi is highly conserved in eukaryotic organisms (Fire, 2007). It is considered as a specific type of defence mechanism (Terenius *et al.*, 2011). Four different types of RNAi have been described including short interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), endogenous siRNAs (endo-siRNAs or esiRNAs), and microRNAs (miRNAs) (Terenius *et al.*, 2011). To date, RNAi has been proven promising for research on gene function determination and gene knockdown in eukaryotes and medical control of cancers and viral disease (Huvenne and Smagghe, 2010).

Widespread increase in the application of RNAi technology in insect research has facilitated the identification of insect gene function. Research has shown that while dsRNA is particularly conservative, there
are various functions and development factors among insect species. Such variations are yet to be fully understood but certainly can serve as a basis for determining their capacity to control insect genes. The main challenge for moving towards larger scale projects remains the development of effective delivery mechanisms. Feeding is very popular in insect RNAi research and may have the most promising future in pest control, especially with the creation of transgenic plants producing dsRNA. Overtime, the use of transgenic insects will also lead to more efficient pest control (Guang Yang et al., 2011).

The present study data was examined with respect to (a) trends in the growth of patenting activity (b) organizations/industries active in research, and (c) the focus of research pertaining to present situation. The analysis has been used to indicate the emerging technological opportunities and trends by highlighting the important technological directions and gaps. The gist of results obtained from the analysis is presented here.

**Patent Analysis**

Patent analysis is a unique management tool for addressing the strategic management of the firm’s technology and product or service development process. Translating patent data into competitive intelligence allows the firm to magnitude its current technical competitiveness, to forecast technological trends, and to plan for potential competition based on new technologies (Fleisher et al., 2003). Patent analysis is an extremely versatile tool, with many implications for businesses strategic planning, mergers, acquisitions, licensing opportunities, R&D management, human resources, competitive intelligence, business intelligence, etc. Since this study was developed as part of an intelligence studies course, my primary aim is to examine patent analysis as a tool for intelligence particularly competitive intelligence. Analytical tools are used for searching patent and associated with scientific literature to show the gap in research areas.

**RNAi insect pest management**

RNA interference (RNAi), the sequence-specific suppression of gene expression, offers great opportunities for insect science, especially to analyze gene function, manage pest populations, and reduce disease pathogens. At present, we have a limited capacity to predict the ideal experimental strategy for RNAi of a particular gene/insect because of our incomplete understanding of whether and how the RNAi signal is amplified and spread among insect cells. Consequently, development of the optimal RNAi protocols is a highly empirical process.

This limitation can be relieved by systematic analysis of the molecular physiological basis of RNAi mechanisms in insects. An enhanced conceptual understanding of RNAi function in insects will facilitate the application of RNAi for dissection of gene function, and to fast-track the application of RNAi to both control pests and develop effective methods to protect beneficial insects and non-insect arthropods, particularly the honey bee (*Apis mellifera*) and cultured Pacific white shrimp (*Litopenaeus vannamei*) from viral and parasitic diseases (Scott et al., 2013). From figure 1 it can be seen that the first patent was initiated in the year 2000 even though the technology was well described by Fire et al. in 1998. It can be seen that there is a gradual increase in the number of patents during 2000 to 2013. The number of patents
reached maximum of 117 in the year 2011 and 2012. In the present year the number of patents granted is 114 till 25.10.2013.

The patent claiming Methods for controlling pests using RNAi, EP2347759 describes the methods for controlling pest infestation using double stranded RNA molecules. This invention also provides methods for producing transgenic cells expressing the double stranded RNA molecules, as well as compositions and commodity products containing or treated with such molecules (Els Van Bleu et al., 2008).

Comparison of Patenting Activity by the Major Assignees

Patent analysis in terms of level of activity gives general information on companies that are active in R&D of these bio-insecticides. The assignees across the globe are shown in figure 2 and following observations were made. (i) Monsanto and syngenta companies hold the maximum number of patents 26.09% and 24.47% respectively, followed by Divergene representing 13.46%. (ii) Among agricultural universities and research Institutions University of California (1.92%) has the maximum number of patents followed by University of Georgia, University of Copenhagen and finally University of Dortmund. Most of the assignees concentrate on the RNAi as the alternative strategy for the effective pest managements. In conclusions the search and analysis of patents drives research strategy and support innovation. With this in mind patent analysis was done to find industrial trend and to understand the competitiveness across the globe. It also provides an insight into emerging technologies and key areas of R&D.

Among current assignee evolution it has been observed that the Monsanto has the maximum and consistent evolution followed by Basf Crop, Dow Agroscience and Bayer crops in RNAi insect pest management R & D activities (Fig. 3).

RNAi across insect orders

Lepidopterans, Coleopterans and Sap sucking pests are the major pests of agricultural crops, and it is becoming increasing urgent to find new methods to control them. From the figure 4A, B and C it has been observed that the patenting trend there is a gradual increase in patents over the period of time. The maximum number of patents were observed in the order Lepidoptera followed by Coleoptera and Hemipteran pests. This data shed lights on the R & D gaps in RNAi insect pest management strategies. This basic information helps in identifying the research gaps and brings out a scope for generating intellectual property through focused research since considerable agricultural crop losses incurred by different insect pest orders where in effective control measures are not available so far.

RNAi transgenic plants

RNA interference (RNAi) is a sequence specific gene silencing mechanism, triggered by the introduction of dsRNA leading to mRNA degradation. It helps in switching on and off the targeted gene, which might have significant impact in developmental biology. Discovery of RNAi represents one of the most promising and rapidly advancing frontiers in plant functional genomics and in crop improvement by plant metabolic engineering and also plays an important role in reduction of allergenicity by silencing specific plant allergens.
Fig. 1 Patenting activity in the field of RNAi in insect pest management trend over the years (1970-2013). IP laws vary between countries and patents have national boundaries, it is essential to perform an FTO IP analysis for each country in which products will be developed and deployed.

Fig. 2 Patenting activity in the field of RNAi in insect pest management major assignees involved in the R & D research.
Fig. 3 Patenting activity in the field of RNAi insect pest management active against different insect orders. Maximum number of patents observed in the order Lepidoptera following Coleoptera and Hemiptera, indicating that emphasis must given to other insect orders also.
Fig. 4. Patenting activity to RNAi insect pest management transgenic plant-based methods for plant pests. A-Patenting trend over the years, B-Major assignees involved in the development of RNAi based transgenic plants and C-major assignees holding the number of patents.
In plants the RNAi technology has been employed successfully in improvement of several plant species by increasing their nutritional value, overall quality and by conferring resistance against pathogens and diseases (Ali et al., 2010). The patenting activities in this field over the years depicted in Fig. (4). It can be observed that the year 2009-2011 has the maximum patenting activity. It is also observed that there was increasing trend in the number of patents since 2004 to 2013. This indicates that the patenting activity has been very recent and that this area has gained importance in recent years. The application of RNA-mediated gene suppression to produce GM organisms evolved from strategies based on expression of target genes in antisense orientation, to co-suppression by over expressing sense transcripts and then to producing dsRNA. There now exist both emerging as well as expiring patents in the United States for the general use of RNAi in plants, and DNA constructs that mediate dsRNA production (Chi-Ham et al., 2010).

RNAi is continuing to develop as a fundamental tool in both plant and animal biotech and an ongoing assessment of the patent landscape will be important to equip scientists and investors with knowledge for evaluating freedom to operate (FTO) in this technology sector (Chi-Ham et al., 2010). Widespread increase in the application of RNAi technology in insect research has facilitated the identification of insect gene function. Research has shown that while dsRNA is particularly conservative, there are various functions and development factors among insect species. Such variations are yet to be fully understood but certainly can serve as a basis for determining their capacity to control insect genes. The main challenge for moving towards larger scale projects remains the development of effective delivery mechanisms. Feeding is very popular in insect RNAi research and may have the most promising future in pest control, especially with the creation of transgenic plants producing dsRNA. Overtime, the use of transgenic insects will also lead to more efficient pest control (Guang Yang et al., 2010). In conclusion analyzing the RNAi insect pest management patents on various criteria can provide valuable information which can be put into use in different ways. These indexes can be used to analyze up-to-date trends of technologies and identifying promising venues for new product development. Patent search and analysis of technological strategies of formulation may lead to higher probability of success in new technological ventures. Finally the specific goal of RNAi insect pest management patent analysis is to discover, develop, and understand new products and methods for safe and effective control of pests, thereby maximizing food production and public health. It has also been observed that patenting activities in the emerging fields of technology are increasingly growing. This quickens the technology evaluation process for businesses and helps to highlight the important technological directions and gaps for further R&D decisions with greater confidence.

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References


PLANT MEDIATED RNAi: A NEW LINE OF DEFENSE AGAINST INSECT PESTS

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Abstract- RNA interference (RNAi) is a potent tool in silencing of genes with high specificity in both invertebrates and vertebrates, thus, it has been widely applied to analyze gene function in various organisms including the insects. This high specificity of gene silencing has potential applications in insect pest management. Recent studies, however, have indicated that specific inhibition of gene expression in insects can also be attained by feeding and topical application of double stranded RNA (dsRNA) in certain insect species. The specific gene silencing using RNAi with feeding and in vivo dsRNA delivery methods holds outstanding promise of application of RNAi for controlling both agriculturally and medically important insect pests. Indeed, transgenic plants expressing dsRNA of specific genes have already been demonstrated for plant resistance against insect pests. This manuscript highlights the improvements and vistas of RNAi technologies in insect pest management, which in turn provides methods for producing transgenic plants express the dsRNA molecules, as well as pesticide agents and commodity products produced by the inventive plants.

Keywords- dsRNA, gene expression, RNAi, Transgenic plants


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Introduction

RNAi is a tool with applications in virtually all spheres of the molecular biology to consider and in the characterization of gene function. The mechanism can be initiated by the introduction of the dsRNA into the system. Until 1980s, RNAi was unknown, but afterwards this genetic modification tool was called as gene silencing and today popularly known as RNA interference (RNAi). The history of RNAi began when Jeongennon conducted an experiment to over express Chalcone Synthase (CHS-a) gene in petunia plant to intensify its flower color to purplish, which resulted in a white flower due to suppression of CHS-a gene and they called it as 'Co suppression' [1]. Thereafter, Fire and Mello in 1998 demonstrated and elucidated details of the RNAi mechanism by the introduction of dsRNA into the Caenorhabditis elegans. After that a spate of publications were appeared, including those of mechanism of dsRNA uptake in insects [2], structural components and function of DICER and RISC etc. [3-5]. Due to its high sequence specificity, it is widely employed in many areas of biology, especially in crop improvement, metabolic engineering, functional genomics, and pest control [6].

Plant pests are the major biotic stress factors decreasing crop productivity both qualitatively and quantitatively to a great extent, control measure using chemical insecticides has resulted in escalation of production cost along with issues of residues leading to and water pollution and more importantly contributed the environmental issues as well as health hazards. The repeated use of chemical pesticide has broken out pest resistance [7]. The evolution of the Bacillus thuringiensis (Bt) toxin transgenic has been utilized for the past one decade in the pest management. The Bt transgenic plants scored a spectacular success in managing many insect orders of agricultural importance viz., Coleoptera, Lepidoptera, etc. but due to continuous Bt cultivation it has resulted in the development of resistance [8]. Lu, et al. [9] described the outbreak of pest the myriad bug and the Bt technology is met with limited success/ ineffective against some of sap sucking pests viz., white fly, aphid, grasshopper. So adding RNAi technology with Bt transgenic either separately or in combination will have added advantages in enhancing the plant protection against insects.

RNAi in Insects

The discovery of RNA interference was first studied in Caenorhabditis elegans [10]. The soaking of nematode in dsRNA solution demonstrated the degradation of the target mRNA and repression of the gene expression. The method of dsRNA application was varied in animals, especially in insects. In C. elegans, soaking and
microinjection methods were preferred and in insects all the methods, including the incorporation of dsRNA synthesizing bacteria in the artificial diet were used, the effect of RNAi was systemic and transitive, the amplification and spread of the RNAi pathway signal occurs through RNA-dependent RNA polymerase [11]. But in insects presence of RDRP is yet to be ascertained, recently RDRP orthologues were characterized in D. melanogaster. In C. elegans turn over of the siRNA molecules occurs through RDRP however, it doesn't require RNAi core elements in the production of siRNA and also siRNA amplification are not necessary for the systemic spread of RNAi. Recently in C. elegans two types of dsRNA mechanism had characterized, one is the transmembrane channel mediated dsRNA uptake mechanism, in this mechanism two types of proteins, namely SID1 (Systemic RNAi Defective) and SID2 are involved, SID1 is required for the systemic spread of RNAi signal and SID-2 is found in the gut tissues and in cooperation with SID1 enhances the environmental RNAi [12-15]. In Drosophila endocytosis mediated dsRNA mechanism has been characterized, the Drosophila cells don't have neither robust systemic RNAi nor SID orthologs but S2 cells when soaked in dsRNA solution showed environmental RNAi, which indicated the possible existence of other alternative mechanism i.e., endocytosis mediated dsRNA uptake [16].

So far in more than 32 insects target genes have been screened for their potential for use in gene silencing mediated by in vivo synthesized dsRNA, recently reviewed by Zhang, et al. [17]. In other insects the RNAi response varied considerably. This was due to the intrinsic nature of the target gene, its size and tissue type and expression of the gene(s) being used for silencing [18]. The RNAi response in several less studied insect species was robust and was inheritable to the next generation [19,20], whereas in other insect species it is stubborn. For example, Lepidoptera showed a variable silencing response when dsRNA was injected [21]. A number of factors are supporting the susceptibility of insect species to RNAi, apart from DICER, a dicer like DNA/RNAse an extracellular enzyme digests dsRNA, these are secreted in various tissues. Other factors include the element of stability/half life of the dsRNA after the entrance into the hemocoele [22-24]. In Blatella germanica the dsRNA persisted for a longer period and in Drosophila and Lepidoptera microinjection of the dsRNA into the hemolymph enhanced the susceptibility to RNAi. The sequence specificity of RNAi was demonstrated in four different insects using E-subunit of V-ATPase, which demonstrated the high sequence selectivity of RNAi. E-subunit of V-ATPase D. melanogaster, Maduca sexta, Tribolium castaneum Herbst and Acrystosiphon Pismum Harris, when each of the specific dsRNA fed individually to all four insects resulted in the selective silencing and death of the respective host insect without affecting other three insects [25].

Plant Mediated-RNAi (PM-RNAi)

The enormous success of RNAi experiments invited plant biotechnologists to utilize the insect functional genes as an insecticidal ingestible dsRNA produced by the transgenic plants. The era of FM-RNA as new line of defense against insects was began [26-28]. Today there are eleven reports that have been published and are summarized in the below [Table-1].

The first outcome of the experimental validation of the insect gene in the form of insecticidal dsRNA was achieved in various insect orders viz., Coleoptera, Lepidoptera and Hemiptera. The first evidence for insect proof plants of RNAi technology demonstrated by Mao, et al. [28] and Baum, et al. [26] against cotton boll worm, Helicoverpa armigera and corn worm, they opined that transgenic plants expressing ingestible dsRNA will emerge as a potential and alternative tool in insect pest management like Bt transgenics.

A PM-RNAi response to root knot nematode (RKN) was successfully established. The RKN i.e., Meloidogyne species, which severely deplete the harvest in a number of food and fiber crops. It infects more than 1700 host plants. Very few resistance crops against RKN have been identified and in other crops resistant gene loci have not been identified. In cognizance of this problem they identified secretory parasitism protein, i.e., a peptide 16D10, which plays a vital role in the early signaling of RKN-host interactions. 16D10 expressed in sub ventral esophageal gland cells. This protein after infection to the roots, directly act with transcription factor domain of SCARECROW [27] establishes its colony and affects root growth. The in vitro studies conducted revealed that a full length dsRNA16D10 led to a reduction of 93-97% of transcript in the M. incognita and 16D10 peptide of 65-69%, further inoculation of the same dsRNA experiment M. incognita on Arabidopsis roots hindered its reproductive ability and reduced galling formation in the roots both in size and in number. To extend this outcome, two fragments of 16D10 i.e., 41 bp and 271bp used in the engineering of A.thaliana plant to silence four species of Meloidogyne RKN species (M.incognita, M.javanica, M. arenaria and M. hapla), where the peptide shared nucleotide sequence 95-98% homology. The transgenic plants expressing the above fragments showed good growth of roots and showed abundant siRNA accumulation in northern blotting, thereby cut off the parasitic process by silencing the 16D10 transcript of M.incognita. This experiment could be employed for the development of transgenic plants where, natural resistance genes do not exist.

Baum, et al. [26], selected 290 genes from the Western corn Root warm (WCR) cDNA library based on the functional role of the gene in the insect life cycle, these genes are called 'targets', the depletion of any one of target genes in the WCR insect may lead to death. The selected genes were screened and determined the larval competence by incorporating the corresponding dsRNA through artificial diet. After seven days most of the larvae had stunt growth and later died. These observation were recorded for two doses of dsRNA concentration, i.e., 520 ng/cm2 and 720 ng/cm2. Further, they extended their research by reducing the dsRNA concentration to 52 ng/cm2 and 5.2 ng/cm2 to determine lethal concentration, at this concentration RNAi response in the form of death and stunt growth was recorded for 125 bp and 69 bp dsRNA's target genes respectively, 14 potential genes for the RNAi in WCR were characterized. Among the selected 14 genes V-ATPase A, D, E and a-Tubulin were showed immediate RNAi response under low dose condition within 24 hours of dsRNA delivery. Due to the rapid RNAi response the above genes were tested for the other Coleoptera member pests, Southern corn rootworm (SCR; Diabrotica undecimpunctata Howard), Colorado potato beetle (CPB; Leptinotarsa decemlineata) and cotton boll weevil (Anthonomus grandis Boheman) to determine the minimized non target effect. Where nucleotide sequence similarities between WCR and CPB were compared which showed the similarities of only 83% and 79% for V-ATPase A and V-ATPase E, respectively. It was apparent that the RNAi response of WCR dsRNA directed to CPB exhibited same results. The success of insecticide V-ATPase dsRNA in insects applied to the cogent evidence of principle in the maize plant by pressing out the V-ATPase 246 bp gene in the sense and antisense.
orientation, the maize plant on exposure to WCR larvae showed reduced root damage and significantly protected the plants.

Recently, Mi-Rpn7 dsRNA producing target gene of M. incognita was introduced into the soyabean plant through hairy root culture for the nematode pest management [29]. Rpn7 gene maintains the infestactivity of the 2SS proteasome and for cleanup of the short-lived proteins that are produced in the regulation of cell cycle and other cell related processes like, metabolic regulation, signal transduction and apoptosis, the group initially repressed Rpn7 gene by soaking with 408 bp dsRpn7 solution it broke up the motility and reduced the infectivity, when the Mi-Rpn7 was transformed to the soyabean plants through hairy root culture. The above results were repeated and also affected the reproduction. However, the transgenic plants developed were not fully tolerant to M. incognita.

The PM-RNAi has been efficiently utilized in the control of a sucking pest like the green peach aphid Myzus persicae, it is one of the major agricultural sap sucking pest that feeds over 40 different plant families and transmits more than 100 types of plant viruses. Pitino, et al. [29] selected Rack1 (Receptor of Activated Kinase C) and COO2 genes. Rack1 based on the earlier gene silencing results was carried out in the C. elegans [30,31] and Heterorhabditis bacteriophora [32]. Where knockdown of this gene in M. persicae resulted in faulty phenotypes, like lethality in an early stage of development, growth retardation and reduced fecundity and caused sterility. Rack1 is the multifunctional receptor protein and one of the internal components of the circadian clock binds to the various proteins and initiate signal transduction cascades, it also functions in the actin organization. Another target gene of M. persicae MpcO02 a homologous gene of COO2 play a role in the plant host interaction and is expressed predominantly in salivary glands, knockdown COO2 gene showed mortality and improved tolerance to peach aphid in transgenic tobacco plants. Pitino, et al. [29] first investigated by expressing both the dsRNA genes transiently in tobacco plants which resulted in a decreases in both the gene transcript levels in insects. Latter they developed stably expressing transgenic Arabidopsis lines by the floral dip method. The transgenic plant showed tolerance to M. persicae, by reducing survival rate and also resulted in a production of fewer nymphs.

Similarly, Mao, et al. [28] silenced the gossypol detoxifying gene of cotton bollworm in in vivo experiment using artificial diet incorporated dsRNA of CYP450 monoxygenase gene, before the engineering of CYP450 monoxygenase gene in the model plant A.thaliana and Nicotina tabacum. first they confirmed toxicity effects of gossypol on larvae using different concentration of gossypol and observed that gossypol at higher concentration (3 mg/L) led to larval growth retardation. Based on this they anticipated that down regulation of the CYP6AE14 gene could cause gossypol toxicity on larvae due to excessive accumulation of gossypol. The CYP6AE14 expression was high in mid gut and was low in Malpighian tubules, transgenic plants imparted susceptibility to cotton bollworm to gossypol. After validating the CYP6AE14 target gene as a potential target in silencing cotton bollworm in model plants A.thaliana and N. tabacum. Mao, et al. [33] extended their work to express dsRNA of CYP6AE14 in cotton plants. Their work significantly proved that the expression of dsRNA of CYP6AE14 in cotton plants showed resistance to bollworm. In their experimental proof of principle the dsCYP6AE14 ingestion of the transgenic leaves arrested the growth of the second instar larvae, the larval weight loss was two folds compared to control larvae after injection of the transgenic leaves and control leaves respectively and also higher gossypol accumulation was recorded in the larvae fed on transgenic leaf. The down regulation of the CYP6AE14 gene in the midgut of bollworm caused gossypol toxification, which resulted in growth retardation and reduced activities.

Mao, et al. [34] further extended their studies and developed a new cotton plant harboring both membrane permeability enhancer gene 3SS:GHCPI and Cytochrome 450 monoxygenase down regulating genes (3SS:dsCYP6AE14). GhCI1 is the serine protease of the cotton plant and play an important role in plant-insect interactions. The entry of GhCI1 increases easier food absorption in the insect midgut. This newly developed cotton plant showed more tolerance over earlier developed RNAi cotton plant (dsCYP6AE14). The cotton plant developed with cysteine protease GhCI1 allowed the entry of larger food molecules into the midgut of H. armigera, as a result more accumulation of gossypol had occurred in the midgut which retarded the growth due to the arrest of Cytochrome 450 monoxygenase gene (3SS:dsCYP6AE14), also further enhance the susceptibility to H. armigera to virus DcCPV. The result of their experiment proves the potential of RNAi in bollworm management and urges the scientific community to develop double constructs in the efficient utilization of if RNAi in the pest management using different targets.

To elicit RNAi mediated protection to tobacco plants against H. armigera, Zhu, et al. [35] used 20-Hydroxyecdysone gene (HaEcr), EcR is a steroid hormone required for the growth and development, particularly for insect molting and metamorphosis, the initiation of the metamorphosis occurs by 20-Hydroxyecdysone via the 20E-Ecr-USP receptor complex and triggers primary and secondary transcriptional genes for molting and metamorphosis. Repression of the 20-hydroxyecdysone caused deformities in the molting and metamorphosis and lead to death, the experimental group first screened in H. armigera, Bombax mori, and in Spodoptera exigua by bacterially expressing the EcR, USP (Ultraspirecle) in HT115-cells contained L4440 and orally delivered dsRNA-USP (S. exigua and B.mor) [36,37] they found the successful RNAi response and observed increased rate above said deformities. Latter EcR was transformed into the tobacco plants. The tobacco plants expressing dsEcr gene showed improved resistance to Lepidopteran insect H. armigera and S. exigua, where the EcR sequence homology was high among them. The transgenic plants expressing dsEcr leaves on ingestion larvae showed a decreased transcript level after 2 and 4 days, the decrease in the mRNA level interrupted and affected larval molting, pupation and adult emergence.

In another study Zha, et al. [38] demonstrated efficient knockdown of the Hemipteran member Nilapavata lugens by expressing the midgut genes, hexose transporter, trypsin like serine protease and carboxypeptidase. Their experiment successfully demonstrated the utility of RNAi in the control of philom sap sucking insect N. lugens and conveyed the usage of such genes in the control of other sucking pests viz., aphids, whiteflies, plant hoppers and plant bugs. Where, the effect of Bt toxin on these sap sucking pests is limited [39]. N. lugens is one of the major sap-sucking pests of rice crop, it sucks sap from the phloem through its mouth stylet and affects the crop growth by reducing the vigor and tiller number/grains and nutritional quality. In this regard, Zha, et al. [38] first identified elements of the RNAi core machinery Nsid-1 and Naub gene, where Nsid-1 is needed for the dsRNA uptake and RNAi spread between the tissues. [40,41] where as Naub is argonate protein required for
the binding of the small RNAs and for the breakdown of the mRNA. The above migut genes were cloned and transformed into rice plants. All the target genes of dsRNA were produced and were partially processed into siRNA in the transgenic plants, on ingesting *N. lugens* nymph showed depletion in the transcript, highest level of gene silencing was recorded for Nttr1 (61%) and for NihT1 (59.3%) and moderate depletion was observed in trypsin like serine protease (41%). Although gene down regulation occurred in the insect, but no mortality was observed.

By using virus based production of dsRNA in plants, a new type of approach was developed for RNAi in the pest management by *M. sexta* [30]. They named it as ‘plant virus based dsRNA producing system’ (VDPs). Originally described and used against roundworms in the presentation of the transient RNAi response in tobacco plants using tobacco rattle virus [42, 43]. To target *M. sexta* they used VDPs approach. They selected nicotine detoxifying cyp genes which were up regulated during nicotine detoxification, particularly, CYP4B46, CYP4M1 and CYP4M3. They selected above three cyp targets based on the sequence homology where CYP4B46 had 85% to CYP4B45, the other two CYP4M1 and CYP4M3 had 53% similarity. They transformed the entire fragment individually into tobacco plants using VDPs method and CYP4B46 fragment alone transformed into the plants using Agrobacterium mediated transformation method. Interestingly the Agrobacterium mediated gene transformation of CYP4B46 did not have any effect on larval mass gain except transcript repression, similarly they had the same effect in the VDPs agro-infilt rated tobacco leaves with CYP4B46, CYP4M1 and CYP4M3 but VDPs-CYP4M3 ingested larvae had comparatively low mass to the other two fragments.

In another study, successful control of *H. armigera* was achieved by knock-down of the Hormonal regulating transcription factor HR-3 [44], studied the effect of dsRNA on the larvae by providing different fragment of dsRNAHaHR-3 in the artificial diet, this bacterially expressed dsRNA could cause significant mortality in the *in vitro* studies. It is evidenced that within 3-7 days all the four types of dsRNA synthesized *in vitro* successfully silenced the gene, among the fragment HaHR-3-1 and HaHR-3-3 were dominant in silencing effect than HaHR-3-2 and HaHR-3-4, the repression of this protein negatively affected the development of the *H. armigera* which caused the incomplete larval molt and no shedding of the cuticle had occurred, apart from this dsRNA ingested larvae lost its weight. The fragment HaHR-3-1 was resulted as most effective target, employing this fragment a transgenic tobacco plants was engineered to produce HaHR-3-3 dsRNA. Tobacco leaf ingested larvae decreased the transcript level and within seven days larvae were dead with developmental deformities. Overall, these results either by transient or stable mode of gene transformation demonstrate that transgenic plants expressing dsRNA do provide protection against insect pests. The gene silencing technique may be a potential strategy for field insect pest control, but suitable insect target genes must be strictly selected when designing dsRNA transgenic plants [38].

### Table 1: List of Plant mediated RNA interference transgenic plants

<table>
<thead>
<tr>
<th>Target Insect</th>
<th>Insect order</th>
<th>Target Gene for Gene silencing</th>
<th>Fragment size used</th>
<th>Intron used for vector construct</th>
<th>Binary vector type</th>
<th>RNAi Transgenic Plant</th>
<th>Effect of PM-RNAI on Insect</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Helicoverpa armigera</td>
<td>Leptodeta</td>
<td>Cytochrome 450 monooxygenase CYP6AE14</td>
<td>448 BP</td>
<td>-</td>
<td>pBI121</td>
<td>Arabidopsis thaliana,</td>
<td>Enhanced gosoply toxicity in larvae, decreased weight &amp; size of the larvae.</td>
<td>Mao, et al [28]</td>
</tr>
<tr>
<td>Helicoverpa armigera</td>
<td>GST- Glutathione - s-transferase</td>
<td>CYP4B46</td>
<td>448 BP</td>
<td>-</td>
<td>pBI121</td>
<td>Tobacco</td>
<td>Depletion in the transcript level</td>
<td>Mao, et al [28]</td>
</tr>
<tr>
<td>Helicoverpa armigera</td>
<td>Lepidoptera</td>
<td>Cytochrome 450 monooxygenase CYP6AE14</td>
<td>448 BP</td>
<td>-</td>
<td>pBI121</td>
<td>Cotton</td>
<td>Larval growth retarded, insect displayed reduced feeding</td>
<td>Mao, et al [33]</td>
</tr>
<tr>
<td>Western root corn worm</td>
<td>VATPase - A</td>
<td>20-Hydroxyecdysone</td>
<td>482 BP</td>
<td>-</td>
<td>pMON40805</td>
<td>Maize</td>
<td>Reduced feeding, stunted larval growth.</td>
<td>Baum, et al [26]</td>
</tr>
<tr>
<td>Manduca sexta</td>
<td>MeCYP6B46</td>
<td>&gt;300 BP</td>
<td>-</td>
<td>pCAMBIA2300</td>
<td>Tobacco</td>
<td>Incomplete moulting, Loss of weight</td>
<td>Kumar, et al [30]</td>
<td></td>
</tr>
<tr>
<td>Helicoverpa armigera</td>
<td>Nematodes</td>
<td>HaHR-3</td>
<td>&gt;300 BP</td>
<td>-</td>
<td>pCAMBIA2300</td>
<td>Tobacco</td>
<td>Reduced moulting and infestation of host</td>
<td>Xiong, et al [44]</td>
</tr>
<tr>
<td>Meloidogyne incognita</td>
<td>Nematodes</td>
<td>Rpm7</td>
<td>408 BP</td>
<td>CHS- a  intron</td>
<td>p3301 and pSAt6</td>
<td>Soybean</td>
<td>Depletion in the transcript level</td>
<td>Niu, et al [49]</td>
</tr>
<tr>
<td>Nilaparvata lugens</td>
<td>Nematodes</td>
<td>Hexose transporter gene, Carboxypeptidase, Trypsin like serine protease</td>
<td>&lt;600 BP</td>
<td>-</td>
<td>pKANNIBAL</td>
<td>Rice</td>
<td>Adversely affected survival rate and decreased fecundity</td>
<td>Zha, et al [38]</td>
</tr>
<tr>
<td>Myzus persicae</td>
<td>Hemiptera</td>
<td>Hemiptera</td>
<td>300 bp</td>
<td>-</td>
<td>pJawohlR-RNAI</td>
<td>Arabidopsis thaliana,</td>
<td>Enhanced gosoply toxicity in larvae, decreased weight &amp; size of the larvae.</td>
<td>Pitino, et al [29]</td>
</tr>
</tbody>
</table>

### Efficacy of PM-RNAI

The efficacy of the RNAI again depends on increasing the availability of longer dsRNA of insects [28, 30]. The plant expressed dsRNA targeted insect genes in the plants were partially processed into small RNA in the plants before it is being available to the insects.

Two groups have shown the efficiency of gene silencing in insects by increasing the duration of the ingestible dsRNA by silencing the plant DICERS [28, 30]. Four types of plant DICERS have been characterized in Arabidopsis and all of the DICERS have overlapping functions. dcl1 generates siRNA and miRNA by cleaving long dsRNA and other three involved in the siRNA production. To test the availability of long dsRNA to the insect from the plant factory, Mao, et al. [28] employed Arabidopsis triple dcl deficient plant (i.e. dcl2, dcl3 and dcl4) and...
transformed the dsCYPB64 transcript. The transformed plant accumulated longer AtdsCYPB64 transcript and depleted siRNA transcript in the mutant plant as evidenced in northern blotting, further the leaf ingested H. armigera larvae showed much depletion the CYPB64 as compared to normal AtdsCYPB64 plant. Further Kumar, et al. [30] also examined the similar role of triple dcl mutant in maximization of the silencing efficiency in insect by delivering the long intact dsRNA. In their transient expression, the group got 16 combinations of four Nttdc constructs and agro-infiltrated with VIGS construct to the NttdsCYPB64 plant, they could establish that two Nttdc combinations, i.e. Nttdc 1, 3 and 4 and Nttdc 2, 3 and 4 silenced plants doubled the accumulation of intact dsCYPB64 and also the dsCYPB64 ingested larvae reduced the CYPB64 transcript.

Future Prospects for PM-RNAi

Although much advancement has been made in the area of RNAi over the past few years, its potential is not fully utilized in the crop improvement for trait of interest. The complexities of RNAi pathway, the molecular machineries, and how it links up to plant development are yet to be clearly elucidated. Employing RNAi, it would be possible to target multiple genes for silencing using a thoroughly designed single transformation constructs. In the future, RNAi can be applied in the management of several insects pest, although it is a prerequisite to determine the chronological succession of high specificity target genes. It is also essential to a firm the safety of transgenic crops to other non-target organisms including human beings before utilization.

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