Cloning and Characterization Of Mannosidase from Tomato and Capsicum
CHAPTER 3
Cloning and Characterization of α-Mannosidase from Tomato and Capsicum

3.1 Introduction
α-Mannosidase enzyme has been investigated for its role in tissue development, seed germination (Ross et al., 1994; Smith and Gross, 2000; Martinez et al., 2004; Minic et al., 2004; Esteban et al., 2005), and recently in imparting freezing tolerance (Thorlby et al., 2001) to the plants. Mannosidase gene has been cloned from Arabidopsis, wherein it has been associated with formation of complex N-glycans (Strasser et al., 2006) and Saccharomyces cerevisiae where it is known to be involved in catabolism of cell wall components mainly in mannan turnover (Kuranda and Robbins, 1987). Furthermore, the protein encoded by this gene is also known to cleave the short chain oligo-mannose residues (8~10) present in oligosaccharides and glycoproteins (Priya Sethu and Prabha, 1997) in plants. Moreover, the plants are known to contain β-1,4-mannosyl residues (mannans) in abundance mainly in the lignified secondary cell walls of gymnosperms, and as a major seed storage polysaccharides in some plants. The mannans are also widespread in Arabidopsis tissues, and may be of particular significance in both lignified and non-lignified thickened cell walls (Handford et al., 2003).

These encouraging reports prompted us to examine the role of mannosidase in tomato and capsicum and test our proposed hypothesis of the enzyme being involved in ripening and/or softening. Therefore, we cloned and functionally characterized the α-Mannosidase gene. This is the first report of molecular cloning and functional characterization of mannosidase from Tomato and Capsicum.

3.2 Materials
3.2.1 Plant materials
Tomato: Pusa Ruby seeds were procured from IARI seeds counter and used for gene expression and agrobacterium mediated transformation.
Tomato mutants: All the tomato mutants (rin, Nor and Nr) were procured from C.M Rick TGRC (Tomato Genetic resource centre)
Capsicum: California wonder and local varieties were used for purification, characterization and gene expression studies.

3.2.2 Bacterial strains and plasmids
Uni-ZAP XR vector: Lambda vector for library construction
Escherichia coli DH5α: With the following genotype ΦdldacZA M15, recA1, endA1, gyrA96, thi-1, hsdR17 (rK-, mK-) supE44, relA1,deoR, (LacZYA-argF)U19
Agrobacterium tumefaciens (EHA 105): This strain contains L, L-succinamopine Ti plasmid which makes it hyper virulent.
pBI121 vector: Binary vector with GUS for over-expression/antisense studies from clontech.
Cloning and Characterization of α-Mannosidase

**pHANNIBAL:** RNAi cloning vector with PDK intron, procured from CSIRO, Australia.

**pART27:** Binary vector for plant transformation procured from CSIRO, Australia.

**pK7FWG2:** Over-expression vector with GFP fusion, procured from VIB, Belgium.

**pGEM-T Easy vector:** Vector for cloning PCR products obtained from Promega.

### 3.2.3 Media and chemicals

<table>
<thead>
<tr>
<th><strong>SOB media (for 1 Litre)</strong></th>
<th><strong>HTB Buffer (200 ml)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto tryptone</td>
<td>HEPES (10 mM)</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>MgCl₂</td>
</tr>
<tr>
<td>Sodium chloride (250 mM)</td>
<td>KCl</td>
</tr>
<tr>
<td>Potassium chloride (10 ml)</td>
<td>3.728 gm</td>
</tr>
<tr>
<td></td>
<td>Was dissolved in 150 ml of ddH₂O and the pH was adjusted to 6.7 with KOH, then the volume was adjusted to 200 ml filter sterilized with 0.45 μm filter and was stored at 4°C</td>
</tr>
</tbody>
</table>

Adjust the pH the media to 7.0 with NaOH, autoclave it then add 5 ml of pre-sterilized 2 M MgCl₂.

<table>
<thead>
<tr>
<th><strong>YEP</strong></th>
<th><strong>Luria broth (LB)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Yeast Extract</td>
<td>25 g/l</td>
</tr>
<tr>
<td>1% Bacto Peptone</td>
<td></td>
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<tr>
<td>0.5% NaCl</td>
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<table>
<thead>
<tr>
<th><strong>TE (pH 8.0)</strong></th>
<th><strong>20X SSC</strong></th>
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</thead>
<tbody>
<tr>
<td>10 mM Tris-HCl (pH 8.0)</td>
<td>175.3 g NaCl</td>
</tr>
<tr>
<td>1 mM EDTA (pH 8.0)</td>
<td>88.2 g Trisodium citrate</td>
</tr>
<tr>
<td></td>
<td>pH 7.0</td>
</tr>
</tbody>
</table>

80% Glycerol

80 ml ultra pure glycerol and 20 ml water mixed and autoclaved

<table>
<thead>
<tr>
<th><strong>80% Glycerol</strong></th>
<th><strong>X-gal:</strong></th>
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<tbody>
<tr>
<td></td>
<td>20 mg/ml in (DMF) Di methyl formamide.</td>
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<table>
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<tr>
<th><strong>IPTG:</strong></th>
<th>200 mg/ml in H₂O</th>
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<table>
<thead>
<tr>
<th><strong>6X Endo-R</strong></th>
<th><strong>2X CTAB Buffer</strong></th>
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</thead>
<tbody>
<tr>
<td>30% Ficoll 400</td>
<td>2% CTAB</td>
</tr>
<tr>
<td>60 mM EDTA (pH 8.0)</td>
<td>1.4 M NaCl</td>
</tr>
<tr>
<td>0.6% SDS</td>
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<table>
<thead>
<tr>
<th><strong>Antibiotics</strong></th>
<th><strong>Alkaline Agarose 2x Loading Buffer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin 100 mg/ml in water</td>
<td>200 μl of glycerol</td>
</tr>
<tr>
<td>Rifampicin 50 mg/ml in methanol</td>
<td>750 μl of water</td>
</tr>
<tr>
<td>Cefotaxime 250 mg/ml in water</td>
<td>46 μl of saturated BPB (Bromophenol blue)</td>
</tr>
<tr>
<td>Ampicillin 50 mg/ml in water</td>
<td>5 μl of 5 M NaOH</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>10x Alkaline Buffer (per 50 ml)</strong></th>
<th><strong>Column-Loading Dye</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>3 ml of 5.0 M NaOH</td>
<td>50% (v/v) glycerol</td>
</tr>
<tr>
<td>2 ml of 0.5 M EDTA</td>
<td>10% (v/v) 10× STE buffer</td>
</tr>
<tr>
<td>45 ml of deionized H₂O</td>
<td>40% (w/v) saturated BPB</td>
</tr>
</tbody>
</table>
Formaldehyde Gel Loading Buffer
720 μl of formamide
160 μl of 10× MOPS buffer
260 μl of 37% formaldehyde
100 μl of sterile water
100 μl of EtBr (10 mg/ml)
80 μl of sterile glycerol
80 μl of saturated BPB in sterile water

Note: The formaldehyde gel loading buffer is not stable and should be made fresh on the day of use.

10× MOPS Buffer
200 mM 3-[N-morpholino]propane-sulfonic acid (MOPS)
50 mM sodium acetate
10 mM EDTA
Adjust to a final pH of 6.5–7.0 with NaOH
Do not autoclave

10× STE Buffer
1 M NaCl
200 mM Tris-HCl (pH 7.5)
100 mM EDTA

SOC media
0.5% Yeast Extract
2.0% Tryptone
10 mM NaCl
2.5 mM MgCl₂
20 mM MgSO₄
20 mM Glucose

Water saturated phenol
Sodium acetate (pH 5.2) 1.67 ml
EDTA(0.5M) pH 8 2 ml
H₂O 96 ml

0.04% methylene blue
Solution prepared in 0.5 M Na-acetate, pH 5.2

Hybridization buffer 2
1% SDS
2X SSC
10% Dextran sulphate
50% deionized fromamide

Denaturing solution (1 Litre)
87.66 g NaCl
20 g NaOH
add in 800 ml water, dissolve and make up the volume.
Stable up to 3 months

Agrobacterium Induction media
0.5% beef extract
0.1% yeast extract
0.5% Peptone
0.5% Sucrose
2 mM MgSO₄
20 mM acetylsyringone, 10 mM MES, pH 5.6

SM Buffer (per Liter)
5.8 g of NaCl
2.0 g of MgSO₄ • 7H₂O
50.0 ml of 1 M Tris-HCl (pH 7.5)
5.0 ml of 2% (w/v) gelatin
Add deionized H₂O to a final volume of 1 liter

Non-denaturing Acrylamide Gel (5%)
Mix the following in a vacuum flask
5 ml of 10× TBE buffer
8.33 ml of a 29:1 acrylamide–bisacrylamide solution
36.67 ml of sterile deionized H₂O
De-gas this mixture under vacuum for several minutes
Add the following reagents
25 μl of TEMED
250 μl of 10% ammonium persulfate

DEPC-treated water (1 litre)
1 ml of DEPC is added and mixed by inverting and incubated overnight. Next day it was autoclaved and used.

RNA Extraction buffer (50 ml)
Water 40.67 ml
3M Sodium acetate (pH 5.2) 3.3 ml
SDS(10%) 5 ml
EDTA(0.5M) pH 8 1 ml

10 M LiCl (100 ml)
42.3 g was dissolved in 60 ml water and volume made up to 100 ml.

Hybridization buffer
0.5M Phosphate buffer, pH 7.2
7% SDS,
1 mM EDTA, pH 8.0

PNP-Alpha-D- Mannopyranoside
FW 301.3
6 mg is dissolved in 100 μl of DMSO and volume made up 1 ml.

Neutralization solution (1 Litre)
87.66 g NaCl
60.5 g Tris
pH adjusted to 7.5 with Conc. HCl

Infiltration medium
10 mM MgCl₂
10 mM MES
200 mM acetylsyringone pH 5.6
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<table>
<thead>
<tr>
<th><strong>15% Urea /Acrylamide (100ml)</strong></th>
<th><strong>Small RNA Hybridization buffer</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>42 g Urea</td>
<td>50% formamide,</td>
</tr>
<tr>
<td>37.5 ml of 40% Acrylamide</td>
<td>7% SDS</td>
</tr>
<tr>
<td>volume made up to 100 ml</td>
<td>50 mM Na₂HPO₄ and NaH₂PO₄ pH 7.2</td>
</tr>
<tr>
<td></td>
<td>Hybridized at 40 °C.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Toluidine Blue O</strong></th>
<th><strong>Calcofluor stain</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05% toluidine blue O in 0.1 M phosphate buffer (pH 6.8)</td>
<td>0.1% calcofluor in water</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>CTAB Buffer</strong></th>
<th><strong>Chloroform: isoamyl alcohol (24:1)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>2% CTAB</td>
<td>24 parts of chloroform and 1 part of iso amyl alcohol mixed in V/V ratio.</td>
</tr>
<tr>
<td>1.4M NaCl</td>
<td></td>
</tr>
<tr>
<td>20mM EDTA, pH 8.0</td>
<td></td>
</tr>
<tr>
<td>100mM Tris-HCl, pH 8.0</td>
<td></td>
</tr>
<tr>
<td>100mM β-ME</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Geraniol (5mM)</strong></th>
<th><strong>ACC (10 mM)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>FW 154.3</td>
<td>FW 101.1</td>
</tr>
<tr>
<td>Density 0.889</td>
<td>1mg in 1ml of water is 10 mM</td>
</tr>
<tr>
<td>Purity 98%</td>
<td></td>
</tr>
<tr>
<td>88.5 μl in 20 ml DMSO and volume made up to 100 ml</td>
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</tbody>
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<table>
<thead>
<tr>
<th><strong>Germination media (1 Litre)</strong></th>
<th><strong>Regeneration/ shooting media</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>30 gm of Sucrose</td>
<td>30 gm of Sucrose</td>
</tr>
<tr>
<td>4.4 gm MS Basal Salt (5519)</td>
<td>4.4 gm MS Basal Salt (5519)</td>
</tr>
<tr>
<td>pH adjusted to 5.6-5.8</td>
<td>pH adjusted to 5.6-5.8</td>
</tr>
<tr>
<td>0.8 gm agar</td>
<td>0.8 gm agar</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Rooting media</strong></th>
<th><strong>Northern washing solution</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>30 gm of Sucrose</td>
<td>First wash</td>
</tr>
<tr>
<td>4.4 gm MS Basal Salt (5519)</td>
<td>2X SSC and 1 % SDS</td>
</tr>
<tr>
<td>100 μl of IAA (10 mg/ml)</td>
<td>Second wash</td>
</tr>
<tr>
<td>pH adjusted to 5.6-5.8</td>
<td>0.5 X SSC and 0.1 % SDS</td>
</tr>
<tr>
<td>0.8 gm agar</td>
<td></td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th><strong>2 XL media</strong></th>
<th><strong>6X Xylene Cyanol</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Yeast extract</td>
<td>0.25 % Xylene cyanol</td>
</tr>
<tr>
<td>2% Bacto tryptone</td>
<td>15 % Ficoll</td>
</tr>
<tr>
<td>0.1% NaCl</td>
<td></td>
</tr>
</tbody>
</table>

### 3.3 Methods

3.3.1 **Construction of expression library**

- Isolation of total RNA
- Isolation of mRNA
- First strand synthesis
- Second strand reaction
- Blunting the cDNA termini
- Alkaline agarose gel
- Ligating the EcoR I adapters
- Phosphorylating the EcoR I ends
- Digesting with Xho I
- Size fractionation and cDNA processing
- Ethidium bromide plate Assay
- Ligation of the fragments
- Packaging reaction
3.3.1 Construction of expression library
Capsicum expression library was constructed using ZAP-cDNA® Synthesis kit and ZAP-cDNA Gigapack III gold cloning kit (Stratagene). For this, isolation of high quality mRNA and reverse transcribing it to cDNA is of prime importance which decides the quality of the library.

3.3.1.1 Isolation of high quality RNA
For capsicum library construction, RNA was isolated using TriPure isolation reagent (Roche Applied Science).

1. During isolation of RNA, enough precautions were taken to prevent RNase contamination, like wearing clean disposable gloves at all the times, using sterile disposable plastic ware and tips reserved for RNA work only and DEPC (Diethylpyrocarbonate) treated water for preparing solutions used in RNA isolation.
2. 0.5 g of tissue was ground in liquid nitrogen and added to 1.0 ml of TriPure reagent and incubated for 1-5 min at room temperature to ensure the complete dissociation of nucleoprotein complexes.
3. Chloroform was added to the samples. For every one ml of TriPure 0.2 ml of chloroform was added and vortexed vigorously for 15 seconds. Then it was incubated for 15 min at room temperature.
4. The tubes were centrifuged at 12,000 g for 15 min at 4°C to separate into three phases. The upper colourless phase was transferred to new tube.
5. Then RNA from the colourless aqueous phase was precipitated with 0.5 ml of isopropanol and inverted several times to mix it thoroughly. Further it was incubated for 5-10 minutes at room temperature.
6. The samples were then centrifuged at 12,000 g for 10 minutes at 4°C and the supernatant was discarded.
7. One ml of 75% ethanol was added to each centrifuge tube and RNA pellet was washed by centrifuging at 7500 g for 5 min at 4°C.
8. The excess ethanol from the RNA pellet was removed and air-dried. Then the pellet was resuspended in DEPC water. Further the RNA was checked on 1.2% denaturing gel.

3.3.1.2 Isolation of mRNA
The mRNA was isolated using poly A tract mRNA isolation systems (Promega)

1. First step in isolation of mRNA is annealing of the oligo dT probe. In a sterile, RNase-free 3ml tube, 1-5 mg of total RNA was taken and the volume was made up to 2.43ml with RNase-free water.
2. The tube was placed in a 65°C heating block for 10 minutes and to it 10μl of the Biotinylated-Oligo (dT) Probe and 60μl of 20X SSC was added. The tube was mixed gently and incubated at room temperature until it cooled completely.
3. While this solution was cooling, stock solutions of 0.5X and 0.1X SSC were prepared. Then the magnetic beads, referred as SA-PMPs (Streptavidin-Paramagnetic Particles), were completely resuspended in order to ensure adequate performance. Discard particles that appear to have “clumped” and cannot be dispersed. To determine if the particles are in good condition, the tube was mixed by inverting several times and verified that the particles remain in suspension for at least 3 minutes in a 0.5 ml volume. If some of the particles settle out of suspension within 3 minutes, forming an easily visible pellet, they should not be used.

4. One tube (3.0 ml volume) of the Streptavidin-Paramagnetic Particles (SA-PMPs) were resuspended per isolation by gently flicking the bottom of the tube until they were completely dispersed, then they were captured by placing the tube in the magnetic stand until the SA-PMPs were collected at the side of the tube (approximately 30 seconds). Then the supernatant was carefully removed and the beads were washed three times with 0.5X SSC (1.5ml per wash), each time capturing them using the magnetic stand and carefully removing the supernatant.

5. Finally they were resuspended in 0.5ml of 0.5X SSC. The entire contents of the annealing (RNA and the Oligo dT from step 1) reaction of the tube and the washed SA-PMPs were incubated at room temperature for 10 minutes by gently mixing and inverting every 1-2 minutes. The SA-PMPs beads were captured using the magnetic stand and carefully supernatant was removed without disturbing the SA-PMP pellet.

6. The particles were washed four times with 0.1X SSC (1.5ml per wash) by gently flicking the bottom of the tube until all of the particles are resuspended. After the final wash, the supernatant was removed without disturbing the SA-PMP particles. Finally the SA-PMP pellet was resuspended in 0.1ml of RNase-free water. Then the SA-PMPs were captured and the mRNA was transferred to a new RNAase free tube.

3.3.1.3 First Strand Synthesis

The ZAP-cDNA synthesis kit uses a hybrid oligo (dT) linker-primer that contains an Xho I restriction site. The outline of the cDNA synthesis protocol is provided in the Figure 3.1.

1. The quality and quantity of the mRNA used is of fundamental importance in the construction of a large, representative cDNA library. For synthesis of first strand cDNA, the water bath was preheated to 42°C.

2. The radioactive [α-32P] dNTP was thawed (do not use [32P] dCTP at all) along with non enzymatic first-strand components. The contents were briefly vortexed, spun down, and placed on the ice. For the control reaction, 25 μl (5 μg) of test RNA and 11 μl of DEPC treated water was added to the RNAase free microcentrifuge. And for the sample reaction following reagents were added in the order to a RNase-free microcentrifuge tube 5.0 μl of 10X first-strand buffer
3.0 μl of first-strand methyl nucleotide mixture
2.0 μl of linker-primer (1.4 μg/μl)
X μl of DEPC-treated water
1.0 μl of RNase Block Ribonuclease Inhibitor (40 U/μl)

3. To the above reaction mixture poly(A) RNA (5 μg) was added gently and incubated at room temperature for 10 min to allow primer annealing. During the incubation, 0.5 μl of the [α-32P]dNTP (800 Ci/mmol) was aliquoted into a separate tube for the first strand control reaction.

4. 3 μl of AccuScript RT was added to the first-strand synthesis reaction. The final volume of the first-strand synthesis reaction should be 50 μl. The sample was mixed gently and the contents were spun down using microcentrifuge.

5. Five μl of the first strand synthesis reaction was aliquoted to the tube containing 0.5 μl of [α-32P]dNTP (800 Ci/mmol). This radioactive sample was the first strand synthesis control reaction.

6. The first strand synthesis reaction and the control reaction were incubated at 42°C for one hour. Meantime a 16°C water bath was arranged for second strand synthesis. After 1 hour, the first strand synthesis reaction was removed from the 42°C water bath.

7. The non-radioactive first-strand synthesis reaction was kept on ice and the radioactive first strand synthesis control reaction was kept at -20°C until it was resolved by electrophoresis on an alkaline agarose gel to test the synthesis of 1st strand cDNA.

3.3.1.4 Second strand reaction
All the non enzymatic second-strand components were thawed on ice and the contents were mixed briefly. The following components were added in order to the 45-μl non-radioactive, first-strand synthesis reaction from the previous step

20.0 μl of 10X second-strand buffer
6.0 μl of second-strand dNTP mixture
114.0 μl of sterile distilled water (DEPC treated water is not required)
2.0 μl of [α-32P]dNTP (800 Ci/mmol)

Then the following enzymes were added to the second strand synthesis reaction:

2.0 μl of RNase H (1.5 U/μl)
11.0 μl of DNA polymerase I (9.0 U/μl)

1. The contents of the tube were gently vortexed and spun in a microcentrifuge. Then the reaction was incubated for 2.5 hours at 16°C.

2. The water bath was occasionally checked to ensure that the temperature does not rise above 16°C. Temperatures above 16°C can cause the formation of hairpin structures, which are unclonable and interfere with the efficient insertion of correctly synthesized cDNA into the prepared vector.
3. After the second strand synthesis for 2.5 hours at 16°C, the tubes were immediately placed on ice.

### 3.3.1.5 Blunting the cDNA Termini

The following contents were added to the second strand synthesis reaction:

- 23.0 µl of blunting dNTP mix
- 2.0 µl of cloned *Pfu* DNA polymerase (2.5 U/µl)

1. The contents were quickly vortexed and the reaction was incubated at 72°C for 30 minutes. Care was taken not to exceed 30 min. Meanwhile the 3 M sodium acetate was thawed.
2. After removing the reaction from 72°C incubation 200 µl of phenol–chloroform [1:1 (v/v)] was added and the mixture was vortexed.
3. The reaction was centrifuged at the maximum speed for 2 minutes at room temperature and the upper aqueous layer containing the cDNA was pippeted out into a new tube.
4. Equal volume of chloroform was added to the tube and vortexed. The reaction was centrifuged at a maximum speed for 2 minutes at room temperature and the upper aqueous layer, containing the cDNA was transferred to a new tube.
5. Then the cDNA was precipitated by adding the following to the aqueous layer:
   - 20 µl of 3 M sodium acetate
   - 400 µl of 100% (v/v) ethanol
6. The reaction was then vortexed and precipitated overnight at −20°C. Next day, in order to orient the direction of precipitate accumulation, the microcentrifuge tube was marked and placed in such a way that the pellet accumulation is towards the tube hinge away from the center of the microcentrifuge.
7. The tube was spun at maximum speed in a microcentrifuge for 60 minutes at 4°C. Care was taken not to disturb the pellet. Further, the pellet was washed by adding 500 µl of 70% (v/v) ethanol to the side of the tube away from the precipitate and centrifuged at maximum speed for 2 minutes at room temperature with the orientation marked.
8. The ethanol was aspirated and the pellet dried by vacuum. Then the pellet was resuspended in 9 µl of *EcoR I* adapters and incubated at 4°C for at least 30 minutes to allow the cDNA to resuspend.
9. One µl of this second strand synthesis reaction was transferred to a separate tube. This radioactive sample is the second strand synthesis control reaction. The protocol recommends resolving the samples of the first and second strand synthesis reactions on an alkaline agarose gel at this point. This helps to determine the size range of the cDNA and the presence of any secondary structure.
3.3.1.6 Alkaline agarose gel
Alkaline agarose gels cause DNA to denature and can be used to identify the presence of a secondary structure called hairpinning. Hairpinning can occur in either the first- or second-strand reactions when the newly polymerized strand "snaps back" on itself and forms an antiparallel double helix. Denaturing gels such as alkaline agarose gels can reveal this secondary structure and can demonstrate the size range of the first and second strand cDNA.

1. 0.8% agarose gel was prepared in 10 X alkaline buffer and the samples were loaded using 2X loading buffer and the gel was run in 1X alkaline buffer at 100 mA.
2. When the BPB (Bromophenol Blue) migrated to ⅓ of the gel, it was sealed in an autoclavable bag and exposed to the film.

3.3.1.7 Ligating the Eco R I Adapters
The following components were added to the tube containing the blunted cDNA and the EcoR I adapters:

- 1.0 µl of 10X ligase buffer
- 1.0 µl of 10 mM rATP
- 1.0 µl of T4 DNA ligase (4 U/µl)
1. The reaction was spun down in a microcentrifuge and incubated it overnight at 8°C. Alternatively, the ligation can also be incubated at 4°C for 2 days. On the next day the ligase is heat-inactivated by placing the tubes in a 70°C water bath for 30 minutes.

3.3.1.8 Phosphorylating the Eco R I Ends
1. After inactivating the ligase, the reaction was spun in a microcentrifuge for 2 seconds. The reaction was cooled to room temperature for 5 minutes. The adapter ends were phosphorylated by adding the following components:
- 1.0 µl of 10× ligase buffer
- 2.0 µl of 10 mM rATP
- 5.0 µl of sterile water
- 2.0 µl of T4 polynucleotide kinase (5 U/µl)
2. The kinase was heat-inactivated at 70°C for 30 minutes. The contents were spun down in a microcentrifuge for 2 seconds and the reaction was allowed to equilibrate to room temperature for 5 minutes.

3.3.1.9 Digesting with Xho I
The following components were added to the above reaction:

- 28 µl of Xho I buffer supplement
3 μl of Xho I (40 U/μl)
The reaction was incubated at 37°C for 1.5 hours.

1. Five μl of 10× STE buffer and 125 μl of 100% (v/v) ethanol were added to the microcentrifuge tube. The reaction was precipitated overnight at -20°C. Following precipitation, the reaction was spun in a microcentrifuge at maximum speed for 60 minutes at 4°C.

2. The supernatant was discarded and the pellet was dried completely. The pellet was further re suspended in 14 μl of 1× STE buffer. To this 3.5 μl of the column loading dye was added. Then the sample was ready to be run through a drip column containing Sepharose CL-2B gel filtration medium.

3.3.1.10 Size fractionation, loading, sample collection and cDNA processing

1. The sepharose CL-2B gel filtration medium and 10X STE buffer was removed from 4°C and equilibrated to room temperature. Further 10X STE buffer was diluted to 1X. For fractionation 1-ml disposable pipet was used. The mouth of the pipet was sealed with cotton plug and the connecting tube was used to attach a 10 ml syringe to the pipet without any gap between the two.

2. The syringe was used as the reservoir for the drip column, further the column was loaded with a uniform suspension of sepharose CL-2B gel filtration medium without trapping any air bubble.

3. The column, after packing, was washed with 10 ml of 1X STE buffer and watched for uniform and steady flow rate.

4. The cDNA was loaded with a pipet when 50 μl of the STE buffer was remaining above the surface of the CL-2B resin. The sample was released gently on the surface of the column and when the sample entered the resin the reservoir was filled with the 3ml STE buffer.

5. The dye starts diffusing as the cDNA migrates in the column. Using a fresh microcentrifuge tubes fractions were collected, three drops per tube, in 12 tubes. 8 μl of each of the fraction was removed and were electrophoresed on 5% non-denaturing acrylamide gel to assess the effectiveness of the size fractionation and to determine the fraction which can be used for ligation.

6. The cDNA fractions were extracted with phenol-chloroform (1:1 (v/v)) and spun for 2 minutes at room temperature. The aqueous layer was transferred to new tube and extracted once with chloroform.

7. The upper aqueous layer was transferred to a fresh microcentrifuge tube and two volumes of 100% (v/v) ethanol was added and precipitated overnight at -20°C. Next day the sample was spun at maximum speed for 60 minutes at 4°C. Then the supernatant was removed to another tube and ensured that all the cDNA was in the pellet.
8. The pellet is carefully washed with 200 µl of 80% ethanol (v/v) by centrifuging at maximum speed for 2 minutes. Then the supernatant was discarded, and the pellet was air dried. Counts in the pellet were verified by hand held Geiger counter and the pellet was dissolved according to it. If the counts were < 30 cps, the pellet was dissolved in 3.5 µl of sterile water and if the counts were > 30 cps then dissolved in 5 µl. The amount of DNA is then quantitated using ethidium bromide plate.

3.3.1.11 Ethidium Bromide Plate Assay
An accurate quantitation of DNA can be obtained by UV visualization of samples spotted on EtBr agarose plates. DNA samples of known concentration are prepared for use as comparative standards in this assay.

1. 100 ml of 0.8% (w/v) agarose was prepared using TAE buffer and 10 µl of EtBr from stock solution (10 mg/ml) was added to it.
2. The mixture was swirled and poured into 90 mm petri dishes and allowed to cool and harden and further incubated at 37 °C to dry it completely.
3. Using a known concentration of standard DNA, several serial dilutions were made and each spotted on the surface of agar without forming a bubble.
4. After spotting all the standards, 0.5 µl of the cDNA sample was spotted on the plate and allowed to dry for 10-15 min. Then the concentration of unknown was compared with the standards.

3.3.1.12 Ligation of the fragments
Ligation of the fragments was done in Uni-ZAP® XR system (Stratagene). It combines the high efficiency of lambda library construction and the convenience of a plasmid system with blue white colony selection. The Uni-ZAP XR vector is double digested with EcoR I and Xho I and will accommodate DNA inserts from 0 to 10 kb in length. The Uni-ZAP XR vector can be screened with either DNA probes or antibody probes and allows in vivo excision of the pBluescript® phagemid, allowing the insert to be characterized in a plasmid system. The polylinker of the pBluescript phagemid has 21 unique cloning sites flanked by T3 and T7 promoters and a choice of 6 different primer sites for DNA sequencing.

1. The Uni-ZAP XR vector arms were shipped in 10 mM Tris-HCl (pH 7.0) and 0.1 mM EDTA. During ligation the vector and insert was taken in equal molar ratio in 2.5 µl volume.
2. Two sets of ligations were set up, one for the control and other for the sample as described below
Control reaction

- 1.0 µl of the predigested Uni-ZAP XR vector (1 µg)
- 1.6 µl of the test insert (0.4 µg)
- 0.5 µl of 10x ligase buffer
- 0.5 µl of 10 mM ATP (pH 7.5)
- X µl of water for a final volume of 5 µl
- X µl of T4 DNA ligase (2 Weiss U)

Sample ligation

The following components were added in order

- 1.0 µl of the predigested Uni-ZAP XR vector (1 µg)
- X µl of the sample insert (100 ng)
- 0.5 µl of 10x ligase buffer
- 0.5 µl of 10 mM ATP (pH 7.5)
- X µl of water for a final volume of 5 µl
- X µl of T4 DNA ligase (2 Weiss U)

3. The reaction was incubated overnight at 12–14°C or for 2 days at 4°C.

3.3.1.13 Packaging reaction

Packaging extracts were used to package recombinant lambda phage with high efficiency. Each packaging extract is restriction minus (HsdR– McrA– McrBC– McrF– Mr–) to optimize packaging efficiency and library representation. Ligations should be carried out at DNA concentrations of 0.2 µg/µl or greater, which favors concatemers and not circular DNA molecules that only contain one cos site. DNA to be packaged should be relatively free from contaminants. Polyethylene glycol (PEG), which is contained in some ligase buffers, can inhibit packaging. The volume of DNA added to each extract should be between 1 and 4 µl in order to obtain the highest packaging efficiency (i.e., the number of plaque-forming units per microgram (pfu/µg) of DNA). Increased volume (i.e., >4 µl) will yield more plaque forming units per packaging reaction, but fewer plaque-forming units per microgram of DNA.

1. Appropriate number of packaging extracts were removed from a −80°C freezer and placed them on dry ice.
2. Packaging extract was quickly thawed by holding the tube between the fingers until the contents of the tube just begins to thaw.
3. The experimental DNA was immediately (1–4 µl containing 0.1–1.0 µg of ligated DNA) added to the packaging extract. Then the contents were stirred with pipet tip to mix well, only gentle pipetting can be done without introducing the air bubbles.
4. The tube was quickly spun (for 3–5 seconds), if desired, to ensure that all contents are at the bottom of the tube. Then the tube was incubated at room temperature (22°C) for 2 hours.

5. After the reaction 500 μl of SM buffer and 20 μl of chloroform was added to tube and the contents were mixed gently. The tube was centrifuged briefly to sediment the debris and the supernatant was transferred to a fresh tube. The supernatant containing the phage was ready for titering which can be stored at 4°C for 1 month.

3.3.2 Cloning of α-Mannosidase

3.3.2.1 Degenerate Primer Design and amplification

Degenerate PCR has proven to be a very powerful tool to find "new" genes or gene families. Degenerate PCR is in most respect identical to ordinary PCR, but with one major difference. Instead of using specific PCR primers with a given sequence, we use mixed PCR primers. That is, if we do not know exactly the sequence of the gene that is to be amplified "wobbles" are inserted in the PCR primers where there is more than one possibility. For instance, a protein motif can be back-translated to the corresponding nucleotide motif (Protein -> Sequence). A nucleotide sequence is called degenerate if one or more of its positions is occupied by one of several possible nucleotides. IUPAC ambiguity codes have been assigned to a set of nucleotides as shown in the Figure 3.3. We performed multiple alignments of the known mannosidase sequences from other plant species and identified conserved blocks/motifs, using these we designed few primers for the mannosidase cloning. We also used the peptide sequences, obtained by LC-MS/MS analysis, effectively for designing degenerate primers. Some of the important points that were considered while designing degenerate primers and amplification are

1. The length of the primers should be minimum 20 bp.
2. The degeneracy should be as minimum as possible. Therefore, such a protein motifs have to be chosen which have amino acids with less or least degeneracy.
3. The template should be cDNA which will reduce the complexity.
4. The primer should be PAGE purified and synthesis scale should be double than that of the normal primers. Because the degenerate primer is a mixture of many sets of individual primers, so as to increase the concentration of each primer it has to be synthesized at higher scale.

5. Low annealing temperatures, as low as 40 °C for 10-15 cycles may be tried which may help in amplifying the target.

6. The predicted size of the amplicon should be less than 1000bp and 40-50 cycles of amplifications can be remunerative.

7. Degeneracy should be avoided at the 3' end of the primer, enzymes like Taq DNA polymerase which do not have 3'→5' exonuclease activity should be used. Sometimes hot start PCR also help to avoid non specific amplification.

8. Some of the programmes used for degenerate primer design are

CODEHOP: COnsensus-DEgenerate Hybrid Oligonucleotide Primers
http://bioinformatics.weizmann.ac.il/blocks/codehop.html
Gene Fisher Interactive PCR Primer Design
http://bibiserv.techfak.uni-bielefeld.de/genefisher/

3.3.2.2 Rapid amplification of cDNA ends (RACE)

This is a procedure for amplification of nucleic acid sequences from a messenger RNA template between defined internal site and either the 3' or the 5' end of the mRNA. 3' RACE takes advantages of natural poly(A) tail found in mRNA as the generic priming site for PCR. To amplify the unknown 3' region we used RACE system from Invitrogen. The summary of 3' RACE system is depicted in the figure 3.5A. The procedure is as follows.

1. Five µg of RNA was used in first strand synthesis reaction. 5 µg of RNA or 50 ng of mRNA was combined with DEPC-treated water to a final volume of 11 µl.

2. One µl of 10 µM AP solution was added and mixed gently and reaction was collected by brief centrifugation. The mixture was heated to 70 °C for 10 min and chilled on ice for at least 1 min. Then the following components were added

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X PCR buffer</td>
<td>2 µl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>2 µl</td>
</tr>
<tr>
<td>10 mM dNTP mix</td>
<td>1 µl</td>
</tr>
<tr>
<td>0.1 M DTT</td>
<td>2 µl</td>
</tr>
</tbody>
</table>

3. The contents of the tube were mixed by pipetting in and out. After a brief spin, 1 µl of Superscript™ II Reverse Transcriptase was added in the PCR tube and the tube was incubated in thermal cycler at 42°C for 50 min followed by a temperature of 70°C for 15 min to terminate the reaction.
4. The contents were chilled on the ice and collected at the bottom of the tube with brief centrifugation. For RNA removal, 1 μl of RNase H was added and the tube was incubated at 37°C for 30 min. This cDNA was used further to isolate the specific genes.

5. To amplify the unknown part of the gene, one gene specific primer (Similar to the sense strand) and another adapter specific complimentary primer was used.

To clone the 5' region of the gene, BD SMART™ RACE cDNA Amplification kit from BD Biosciences was used. Primers designed were 25 mer with Tm of more than 65°C and complimentary to the sense strand. This kit uses the switching mechanism at 5' end of RNA transcript for synthesis of full length cDNA (Figure 3.6).

1. We started with 1 μg of mRNA for synthesis of cDNA. In a 0.5 ml microcentrifuge 1 μl of 5'-CDS primer and 1 μl of BD SMART II A oligo along with the mRNA making the volume to 5 μl.

2. The contents were mixed briefly in a microcentrifuge and incubated at 70°C for 2 min and then cooled on ice for 2 min. Following components were added to the tube (Already containing 5 μl).

<table>
<thead>
<tr>
<th>5X First-strand buffer</th>
<th>2 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>DTT (20 mM)</td>
<td>1 μl</td>
</tr>
<tr>
<td>dNTP Mix (10 mM)</td>
<td>1 μl</td>
</tr>
<tr>
<td>BD PowerScript Reverse Transcriptase</td>
<td>1 μl</td>
</tr>
</tbody>
</table>

3. The contents were mixed and spun briefly. Then incubated at 42°C for 1.5 hr in an air incubator or hot lid thermal cycler. After the reaction, the product was diluted with 200 μl of Tricine-EDTA Buffer.

4. The tubes were further heated at 72°C for 7 min and the cDNA stored at -20°C. This cDNA was used to amplify the target using gene specific primer.

5. PCR master mix was prepared which had the following components

<table>
<thead>
<tr>
<th>PCR-Grade water</th>
<th>34.5 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X BD Advantage 2 PCR Buffer</td>
<td>5 μl</td>
</tr>
<tr>
<td>dNTP Mix (10 mM)</td>
<td>1 μl</td>
</tr>
<tr>
<td>50 X BD Advantage 2 Polymerase Mix</td>
<td>1 μl</td>
</tr>
</tbody>
</table>

The components were mixed and spun briefly, then to this 2.5 μl of cDNA, 5 μl of UPM (Universal primer mix) (10X) and 1 μl GSP1 (Gene specific primer 1) (10 μM) was added and amplified in a thermal cycler using touchdown PCR. Further, part of the PCR product was resolved on 1 % agarose gel. One important point to be noted is that the primary PCR may also result in the smear or several bands, therefore it is necessary to go for the secondary PCR with another nested primer (GSP2) to be more specific.
3.3.2.3 Cloning of DNA fragments
The fragments obtained as a result of PCR, were either cleaned with PCR purification kit and ligated to pGEM-T Easy vector or electrophoresed on the 0.8% agarose/EtBr gel, eluted from the agarose gel and ligated to the T-vector.

3.3.2.3.1 Elution of DNA from agarose gel
1. The PCR product was fractionated on 1% agarose/EtBr gel and the band was cut by using sterile blade and collected in a 1.5 ml sterile micro-centrifuge tube. The gel elution was performed by using MinElute gel extraction kit (Qiagen, Germany) according to the manufacturer's instructions with minor modifications.
2. Three volumes of buffer QG was added to the eppendorf containing one volume of gel slice (one volume of gel is 100 mg ~ 100 µl) and incubated at 50°C for 10 min to dissolve the agarose.
3. After the gel slices were dissolved completely, one gel volume of isopropanol was added and mixed by inverting the tubes 4-5 times. Then the sample was loaded onto the MinElute column which was kept on a 2 ml collection tube and centrifuged at 13,000 rpm for 1 min.
4. The flow-through was discarded and the column was again placed in the same collection tube. Further, 500 µl of QG buffer was loaded to the column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and column was again placed in the same collection tube.
5. To wash the column, 750 µl buffer PE was loaded into the column and centrifuged at 13,000 rpm for 1 min. The flow-through was discarded and column was again placed in the same collection tube and centrifuged for an additional 1 min to remove the residual ethanol.
6. The MinElute column was then placed in clean 1.5 ml micro-centrifuge tube. To elute the DNA, 10 µl of elution buffer (100mM TrisCl, pH 8.0) or sterile nuclease free water was loaded directly on the matrix. The column was left as such for 2 min and then centrifuged at 13,000 rpm for 2 min. DNA was obtained as flow through. The eluted DNA was stored at -20°C until use.

3.3.2.3.2 Ligation
Most of the PCR amplified fragments were cloned in pGEM-T Easy vector
1. Vector, ligation buffer were thawed on the ice and contents centrifuged briefly.
2. In a 0.5 ml tube (known to have low-DNA binding capacity) ligation was set by adding 5 µl 2X ligation buffer, 1 µl of pGEM-T Easy Vector (50 ng) and PCR product in a molar ratio of 1:3 (Vector: Insert)
3. Then 1 µl of T4 DNA ligase was added, the contents were mixed and incubated at 4°C overnight. Next day 1-2 µl of the ligation mix was transformed using *E. coli* competent cells.

### 3.3.2.3.3 Preparation of Competent Bacterial Cells

For cloning purpose, *E. coli* DH5α bacterial strain were made competent by the below given methods and used for transformation.

#### 3.3.2.3.3.1 Calcium Chloride Method

The CaCl₂ method was adopted from Sambrook and Russell (2001) with some minor modifications.

1. From the overnight grown pre-culture of bacterial cells, 1ml of inoculum was used to inoculate 100 ml LB medium in a culture flask. This culture was grown at 37°C with vigorous shaking (200-250 rpm) to an *A*₆₀₀ of 0.3-0.4.
2. The culture was chilled on ice for 15-20 min, transferred to pre-chilled SS-34 tubes and centrifuged at 5000 rpm for 5 min at 4°C in Sorvall® RC5C plus centrifuge (Kendro Lab., USA) with SA-600 rotor.
3. The pellet in each tube was gently suspended in 0.5 volumes (of original culture) of ice-cold 100mM CaCl₂ by gently swirling the tubes and incubated on ice for 30 min.
4. The cells were collected by centrifugation as above and resuspended in 0.1 volumes of ice-cold 100mM CaCl₂ by gently swirling the tube. Finally, the cells were dispensed in 1.5ml pre-chilled eppendorf tube and frozen in liquid nitrogen.

#### 3.3.2.3.3.2 Preparation of ultra-competent bacterial cells

The competent cells were prepared as described by Inoue et al. (1990) with few modifications.

1. DH5α bacterial cells were streaked from the glycerol stocks on LB agar plate and were grown overnight at 37°C.
2. Approximately 5-10 large colonies were inoculated in 200 ml SOB media with a sterile loop and grown at 22°C with vigorous shaking at 200-250 rpm till the *OD*₆₀₀ reaches to 0.45. The culture flask was removed from the incubator and placed on ice for 10 min.
3. The culture was transferred to sterile pre-chilled SS-34 centrifuge tubes, and centrifuged at 2500X g for 10 min at 4°C. The pellet obtained was resuspended in 16 ml of ice-cold HTB, incubated on ice for 10 min and centrifuged at 2500x g for 10 min at 4°C.
4. The pellet obtained was gently resuspended in 4 ml of HTB and DMSO was added to a final concentration of 7% with gentle swirling. Cells were kept on ice bath for 10 min.
5. One hundred microliters of the cell suspension was dispensed in 1.5 ml micro-centrifuge tubes and snap-frozen in liquid nitrogen. The frozen competent cells were stored at -80°C for future use.
3.3.2.4 Transformation

Competent *E. coli* cells were transformed according to the standard protocol given by Hanahan, (1983).

1. A vial of competent cells, stored at -80°C was carefully thawed on ice avoiding any temperature shock. The ligated product or plasmid was directly added to 100 μl competent cell suspension, mixed by gentle tapping and subsequently kept on ice for 30 min.
2. The cells were then given a heat shock at 42°C for 90 sec and quick chilled on ice for 5 min. This was followed by addition of 0.9 ml of LB/SOC/2XL and the cells were allowed to grow at 37°C for 60 min with gentle shaking.
3. The transformed competent cells were plated on LB plate containing appropriate antibiotic. Blue-white selection if needed, was carried out by plating the cells on X-gal/IPTG plate. The plates were then incubated at 37°C overnight.

3.3.2.4.1 Presence of the insert

The presence of the insert in the clone was confirmed by the colony PCR by using either gene specific primers or primers compatible with cloning vector.

1. Individual colonies were picked from overnight grown plate and mixed in 20 μl sterile water in a 0.5 ml micro-centrifuge tubes. The cells were lysed by boiling for 2 min and centrifuged at 13,000 rpm for 30 sec.
2. Five microlitre of the supernatant was taken as template for PCR. The master mix was prepared depending on the number of the PCR reactions and distributed in thin-walled PCR tubes. Number of PCR cycles and cycling conditions were adjusted according to the T_m of primers used for amplification. Usually M13 Forward (5’ CGCCAGGGTTTTCCCAGTCACGAC 3’) and M 13 Reverse primers (5’ AGCGGATACACAATTTCACACAGGA 3’) (Universal primers) were used in colony PCR. The components of colony PCR and the cycling conditions used are mentioned below:

<table>
<thead>
<tr>
<th>Components of colony PCR</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 × PCR buffer</td>
<td>2.5 μl</td>
</tr>
<tr>
<td>25 mM MgCl₂</td>
<td>1.5 μl</td>
</tr>
<tr>
<td>M13 F (10 μM)</td>
<td>1 μl</td>
</tr>
<tr>
<td>M13 R (10 μM)</td>
<td>1 μl</td>
</tr>
<tr>
<td>dNTPs (10 mM)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Taq polymerase (5 U/μl)</td>
<td>0.25 μl</td>
</tr>
<tr>
<td>Water</td>
<td>17.75 μl</td>
</tr>
</tbody>
</table>

**Cycling conditions**

<table>
<thead>
<tr>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>2 min</td>
</tr>
<tr>
<td>94°C</td>
<td>1 min</td>
</tr>
</tbody>
</table>
3. If the PCR product is amplified using an enzyme with proof reading activity A-tailing was done to clone the fragment.

3.3.2.4.2 Plasmid isolation

After identifying the positive colonies, they were inoculated in 5 ml of LB with 50 μg/ml of ampicillin and grown overnight at 37 °C. Next day the plasmids were isolated using Wizard® Plus SV Minipreps DNA Purification System

1. 1.5 ml (high-copy-number plasmid) or 10 ml (low-copy-number plasmid) of bacterial culture was harvested by centrifugation for 5 minutes at 10,000 x g in a tabletop centrifuge. The supernatant was discarded and the tubes were inverted on a paper towel to remove excess media.

2. 250 μl of Cell Resuspension Solution was added and the cells were completely resuspended by vortexing or pipetting. It is essential to thoroughly resuspend the cells and transfer them to 1.5 ml microcentrifuge tube.

3. After resuspension 250 μl of Cell Lysis Solution was added and mixed by inverting the tube 4 times (do not vortex). Then the cells were incubated until the cell suspension become clear. (approximately 1-5 minutes).

4. 10 μl of Alkaline Protease Solution was added and mixed by inverting the tube 4 times and incubated for 5 minutes at room temperature. Alkaline protease inactivates endonucleases and other proteins released during the lysis of the bacterial cells that can adversely affect the quality of the isolated DNA.

5. Then 350 μl of Neutralization Solution was added and immediately mixed by inverting the tube 4 times.

6. The bacterial lysate was centrifuged at maximum speed (around 14,000 x g) in a microcentrifuge for 10 minutes at room temperature. Meantime the plasmid DNA purification unit was assembled by inserting one Spin Column into one 2 ml Collection Tube for each sample. Then the cleared lysate (approximately 850 μl,) was transferred to the prepared Spin Column by decanting. Further, the supernatant was centrifuged at maximum speed in a microcentrifuge for 1 minute at room temperature. And the flow through was discarded from the Collection Tube.

7. The Spin column was reinserted into the collection tube and 750 μl of column wash solution was added and centrifuged at maximum speed for 1 minute at room temperature. The wash step was repeated using 250 μl of Column wash Solution.
8. Then the spin column was transferred to a new, sterile 1.5ml microcentrifuge tube and the plasmid DNA was eluted by adding 30μl of nuclease-free water to the Spin Column and centrifuged at maximum speed for 1 minute at room temperature in a microcentrifuge. Further the eluted DNA was stored at -20 °C.

9. The plasmids isolated were checked on the 0.8% agarose gel and were sequenced in ABI (Applied biosystems) 3700 DNA sequencer.

3.3.3 In-silico analysis

3.3.3.1 BLAST
Sequence alignments provide a powerful way to compare novel sequences with previously characterized genes. Both functional and evolutionary information can be inferred from well designed queries and alignments. BLAST 2.0, (Basic Local Alignment Search Tool), provides a method for rapid searching of nucleotide and protein databases. Since the BLAST algorithm detects local as well as global alignments and finds regions of similarity embedded in otherwise unrelated proteins. Both types of similarity may provide important clues to the function of uncharacterized proteins. We performed BLASTN, which searches nucleotide database to nucleotide query, and blastx, which searches protein database using a translated nucleotide query. Both the searches were against the nr (non-redundant) database.

3.3.3.2 ORF finder
The ORF Finder (Open Reading Frame Finder) is a graphical analysis tool which finds all open reading frames of a selectable minimum size in a user's sequence or in a sequence already in the database. This tool identifies all open reading frames using the standard or alternative genetic codes. The deduced amino acid sequence can be saved in various formats and searched against the sequence database using the WWW BLAST server. This can be accessed at NCBI
http://www.ncbi.nlm.nih.gov/gorf/gorf.html. This program was used to find the longest coding region within the mannosidase sequence.

3.3.3.3 Back translation
The nucleotide sequence was converted to protein sequence by translating the DNA sequence in all the six reading frames using the program translate tool in Expasy server http://www.expasy.ch/tools/dna.html to identify the correct reading frame of the sequence. The protein sequence was further analyzed for its other properties described below.

3.3.3.4 Analysis of Protein sequence and the properties
Protein sequence analysis was done using expasy (http://www.expasy.ch/tools/#ptm) tools. For calculating theoretical pi and Mw of the protein Compute pi/Mw

InterPro http://www.ebi.ac.uk/interpro/

InterPro is a database of protein families, domains and functional sites in which identifiable features found in known proteins and can be applied to unknown protein sequences. Basically it finds out the conserved domains in the protein sequence. Another database called the ProDom database (http://prodom.prabi.fr/prodom/current/html/form.php); is part of the InterPro project which can be used for the same purpose.

3.3.3.5 Hydropathy plot and Transmembrane domain prediction
A graph which shows how hydrophobic each amino acid in a polypeptide, is versus where it is located on the polypeptide. It is used to find cluster of hydrophobic amino acids, which could indicate that the polypeptide in question is a transmembrane protein or has transmembrane domain. Kyte-Doolittle is a widely applied scale for delineating hydrophobic character of a protein. Regions with values above 0 are hydrophobic in character. Here choosing the window size is crucial which refers to the number of amino acids examined at a time to determine a point of hydrophobic character. We used the program on ExPASy server (http://expasy.org/cgi-bin/protscale.pl) to calculate the hydrophobicity of the protein. The TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html) makes a prediction of membrane-spanning regions and their orientation. The algorithm is based on the statistical analysis of TMbase, a database of naturally occurring transmembrane proteins.

3.3.3.6 Subcellular localization
TargetP 1.1 predicts the subcellular location of eukaryotic proteins. The location assignment is based on the predicted presence of any of the N-terminal presequences: chloroplast transit peptide (cTP), mitochondrial targeting peptide (mTP) or secretory pathway signal peptide (SP). For the sequences
predicted to contain an N-terminal presequence a potential cleavage site can also be predicted by using this server (http://www.cbs.dtu.dk/services/TargetP/). The method has been tested on A. thaliana and H. sapiens sets. LOCtree is a novel system of support vector machines (SVMs) that predict the subcellular localization of proteins, and DNA-binding propensity for nuclear proteins, by incorporating a hierarchical ontology of localization classes modeled onto biological processing pathways. LOCtree was 74% accurate for non-plant eukaryotes, 70% for plants, and 84% for prokaryotes during six fold cross-validation on a non-redundant data set. This can be assessed at http://cubic.bioc.columbia.edu/cgi-bin/var/nair/loctree/query. SignalP (Bendtsen et al., 2004) was also used to identify the N-terminal signal peptide cleavage in both the capsicum and tomato protein sequence.

3.3.3.7 ClustalW
ClustalW is a general purpose multiple sequence alignment program for DNA or proteins. It produces biologically meaningful multiple sequence alignments of divergent sequences. It calculates the best match for the selected sequences, and lines them up so that the identities, similarities and differences can be seen. Evolutionary relationships can be seen via viewing Cladograms or Phylogenograms. The basic information they provide is the identification of conserved sequence regions. This is very useful in designing experiments to test and modify the function of specific proteins, in predicting the function and structure of proteins and in identifying new members of protein families. The ClustalW web form is available at http://www.ebi.ac.uk/Tools/clustalw/.

3.3.3.8 Phylogenetic analysis
A phylogeny is the evolutionary history of a group of entities. The main aim of phylogeny construction is to describe evolutionary relationships in terms of relative recency of common ancestry.

3.3.3.9 3-D structure prediction
Three dimensional structures of mannosidase from both capsicum and tomato were predicted from comparative homology modeling servers like SWISS MODEL (http://swissmodel.expasy.org//SWISS-MODEL.html) or 3DJIGSAW (http://www.bmm.icnet.uk/servers/3djigsaw/). We used the latter one to predict the possible model for both the proteins. This server builds three-dimensional models for proteins based on homologues of known structure, and the model coordinates are written in PDB format, the most widely used for proteins. In the automatic mode of submission the program looks for homologous templates in our sequence databases (PFAM+PDB+nr) and splits the query sequence into domains. If good templates are found, the best covered domain is then modeled using a maximum of 2. The files can be viewed in programs like RasMol, Deep viewer and PyMOL.
3.3.4 Gene expression analysis

### 3.3.4.1 RNA isolation and quantification
RNA was isolated according to the protocol of Menke et al., (1999)

1. In a SM-24 tube three ml of extraction buffer and three ml of water saturated phenol was added. To this extraction mixture 0.5 mg of tissue was ground in liquid nitrogen and added.
2. Tissue was mixed well by vortexing and centrifuged at 13,000 rpm for 10 min at RT. The aqueous layer was taken and to it three ml of water saturated phenol and 1.5 ml of chloroform was added and vortexed vigorously for 20 sec.
3. Then the tube was centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was precipitated with 1/3rd volume of 10M LiCl, mixed well and incubated at 4°C overnight.
4. Next day it was centrifuged at 10,000 rpm for 15 min at 4°C. The pellet was washed once with 2.5 M LiCl and further the pellet was washed with 70% EtOH at 10,000 rpm for 5 min at 4°C.
5. Then the pellet was dried and dissolved in DEPC water. Further, the RNA was quantified using spectrophotometer (U-2010, HITACHI) against DEPC-treated water as blank. After brief centrifugation, one microlitre of the RNA was diluted 1000 times by adding 1 ml of DEPC-treated water and mixed thoroughly. The O.D of this diluted RNA was taken at 260 nm. Concentration of the RNA was calculated according to the following formula-

\[
\text{RNA conc. (μg / μl): } \frac{\text{O.D}_{260} \times 40 \times \text{Dilution factor}}{1000}
\]

Purity of the RNA was checked by taking O.D at 260, and 280 nm wavelengths. The RNA was indicated as pure if the ratio of O.D (260/280) values were in between 1.7-2.0 (<1.7 is typically protein contamination).

### 3.3.4.2 Isolation of mRNA
The mRNA was isolated using Dynabeads® mRNA purification kit from DYNAL biotech. This is based on the unique dynabeads magnetic separation technology.
1. 200μg of RNA was adjusted to 100 μl with DEPC water. The RNA was heated to 60 °C for 2 minutes to disrupt secondary structure and suddenly chilled.

2. In the meantime 200 μl (1mg) of Dynabeads oligo (dT)25 from the stock tube suspension was taken and dispensed into a 1.5 ml eppendorf tube and placed it on the Dynal MPC magnetic stand for 30 sec. The supernatant was pipetted out and to it 100 μl of binding buffer was added to the magnetic beads.

3. The eppendorf was removed from the magnetic stand and the magnetic particles were resuspended in the binding buffer. To this eppendorf the total RNA (200μg in 100 μl of binding buffer) was added and mixed thoroughly by rotating it on roller or mixer for 3-5 minutes at room temperature.

4. Then the eppendorf was placed on the magnetic stand for 30 sec or until the solution became clear. The supernatant was removed and the beads were washed twice with washing buffer B. Care was taken to remove all the supernatant between the wash.

5. The mRNA was eluted by adding desired amount of 10 mM Tris-CI to the beads and heated to 70°C for 2-3 minutes and the eppendorf was immediately placed on the magnetic stand and the eluted mRNA was transferred to new eppendorf tube. The quantitation was done using Nanodrop spectrophotometer ND 1000 V 3.1.1.

3.3.4.3 Denaturing formaldehyde gel for RNA electrophoresis

1. Total RNA was run in 1.2 % denaturing formaldehyde gel. For preparation of gel, 1.2 g agarose was added to 72ml DEPC treated water and boiled for 15 min.

2. Once the temperature came down to 60°C, 18 ml formaldehyde and 10 ml 10X MOPS buffer was added. The contents were mixed by swirling.

3. The molten gel was poured in casting tray with combs already fitted into it. Meanwhile, RNA samples were prepared by mixing ten microgram of total RNA and RNA loading dye. The samples were heat denatured at 65°C for 10 min and immediately chilled.

4. The samples were run at 20-30 Volts for 5-6 hours in 1X MOPS buffer.

3.3.4.4 Transfer of total RNA to Nylon Membrane

1. The gel was rinsed with DEPC treated water for 30 min to remove formaldehyde and it was equilibrated with 20X SSC for 30 min.

2. The RNA was transferred to Hybond–N’ Nylon membrane (Amersham, UK) by vertical capillary action using 20X SSC for 16 h. After that the RNA was cross-linked to the nylon membrane in UV crosslinker (Stratagene, USA) at 1200kJ/cm² and this RNA cross-linked membrane was treated with 5% glacial acetic acid for 15 min.

3. To check the RNA transfer on the membrane, it was stained with 0.04% methylene blue (Solution prepared in 0.5 M Na-acetate, pH 5.2). Excess of the stain on the membrane was
removed by washing with sterile MQ water. The nylon membrane was air dried and wrapped in a saran wrap.

3.3.4.5 Radioactive probe preparation, purification and hybridization

For probe preparation radiolabeled nucleotide was used, hence all steps were performed in radioactive room taking adequate safety measures.

1. In a hybridization incubator, the RNA cross-linked nylon membranes were incubated at 60°C with 10 ml of pre-hybridization solution (0.15M Phosphate buffer, pH 7.2, 7% SDS, and 1mM EDTA, pH 8.0) in hybridization bottles for 4 hrs or at 42 °C using 1% SDS, 2X SSC, 10% Dextran sulphate and 50% deionized formamide.

2. In the meantime the probe was prepared using random primers labeling NEBlot® kit (NEB Inc., U.K). For probe preparation, in 1.5 ml micro-centrifuge tube 50 ng of DNA (fragment to be used as probe) was taken in final volume of 10 μl.

3. The dsDNA was denatured for 5 min in boiling water bath and quickly chilled on ice. For 50 μl reaction, the following components were added in the order- 26 μl of MQ H₂O, 5.0 μl of 10X labeling Buffer, 2.0 μl of dATP, 2.0 μl of dGTP, 2.0 μl of dTTP, 2.0 μl of radioactive α³²P-dCTP (3000 Ci/mmole, Amersham Biosciences) and 5 units of exo·Klenow polymerase enzyme. The final mixture was incubated at 37°C for one hour in water bath.

4. For purification of free radioactive dNTPs from the mixture, Sephadex G-50 column was prepared as described. One ml fresh disposable sterile syringe was packed at the bottom with the glasswool. This column was packed with sephadex G-50 (soaked in TE, pH 8.0) up to appropriate volume by centrifugation in a 15 ml falcon tube and was equilibrated thrice with TE, pH 8.0.

5. Prior to purification it was centrifuged again, to remove excess TE, at 2,300 rpm for 2 min. The volume of the reaction mix was made upto 200 μl with TE, pH 8.0. The reaction mix was loaded on the packed column and centrifuged at 2,300 rpm for 2 min. Purified probe was collected as flowthrough in a decapped eppendorf and transferred to fresh eppendorf.

6. It was subsequently denatured for 5 min in boiling water bath and quick chilled for 5 min. After a brief spin, the probe was added directly to the pre-hybridization solution kept in hybridization bottle. The probe was left for hybridization for 14-16 hr at 60°C in hybridization incubator.

3.3.4.6 Washing and Autoradiography

1. Filters (Hybridized nylon membrane) were washed thrice for 5 min at room temperature in low stringency solution (2X SSC and 1% SDS). Filters were then checked for the count by the radiation monitor.
2. This was followed by washing at 60°C in medium stringency washing solution (0.4X SSC and 0.1% SDS) for 10 minutes or more depending upon the background count.

3. The filters were then wrapped in saran wrap to avoid drying and the X-ray film was exposed to the membrane in the Hypercassette™ (Amersham Pharmacia biotech) for the time period depending upon the signal intensity.

4. Subsequently, the X-ray film was developed using Developer and Fixer solutions (Kodak Affiliate Products, India). The autoradiograms obtained were scanned in Fluor-S™ Multilumer (Bio-Rad, USA).

3.3.5 Functional characterization of Mannosidase

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3.3.5.1 Construct preparation

3.3.5.1.1 RNAi construct

3.3.5.1.1.1 Cloning in pHANNIBAL vector

In pHANNIBAL (with ampicillin resistance in bacteria) the PCR fragments are inserted into these vectors using conventional restriction enzyme digestion and DNA ligation techniques.

1. PCR amplification of part of the gene (500-600 bp including either 5′ UTR or 3′ UTR) was done incorporating restriction site at both the ends such that the sense strand had XbaI and ClaI and antisense strand had XhoI and KpnI.

2. The PCR product was cleaned and digested with XhoI / KpnI for sense arm cloning and XbaI / ClaI for antisense arm cloning.

3. Digested fragments were sequentially ligated to XhoI / KpnI and XbaI / ClaI digested pHANNIBAL vector. Ligation of both the fragments was confirmed by digesting the plasmid once with each set of restriction enzymes.

4. The pHANNIBAL vector containing sense and antisense strand was digested with NotI to separate the ihpRNA cassette and ligated to the binary vector pART27. Further sequence verification was done by cloning the NotI fragment in pGEM-T vector and analyzed by sequencing.

3.3.5.1.2 Antisense construct

3.3.5.1.2.1 Cloning in pBI 121 vector

Antisense construct was prepared in pBI 121 replacing the GUS gene.

1. Full length mannosidase was amplified with Sac I in forward primer and Xba I in reverse primer. The amplified product was gel eluted and the concentration of the insert was determined by resolving on 1% agarose gel.

2. 500 ng of the vector (pBI 121) was digested with Xba I and Sac I to release the GUS gene, and the double digested product was gel purified.

3. Ligation reaction was set in 10 μl reaction volume, using vector and insert in the ration 1:3 respectively and 0.5 μl of T4 DNA ligase (20,000 Units/ml) in 1X ligation buffer. The reaction was incubated at 4°C for 12-16 hrs.

4. Next day, half of the ligation mix was transformed into ultra competent cells and plated on kanamycin (50μg/ml) plate. The colonies obtained were checked for the presence of the insert as well the orientation by digestion. The positive clone was then transformed to Agrobacterium by electroporation.
3.3.5.1.3 Over-expression construct

3.3.5.1.3.1 Cloning in PK7FWG2 vector

This is a Gateway cloning vector which facilitates directional, recombinational in vitro cloning. The vector is 11880 bp in length and has enhanced GFP in the gene which gets fused to the 3' end of the gene. The backbone of this described GATEWAY™-compatible vector is pZP200 which is relatively small (6.7 kb) plasmid with pBR322 mobilization site (Karimi et al., 2002). The GATEWAY™ conversion technology (Invitrogen, Gaithersburg, MD, USA) is based on the site-specific recombination reaction mediated by phage λ. DNA fragments flanked by recombination sites (att) can be transferred into vectors that contain compatible recombination sites (attB × attP or attL × attR) in a reaction mediated by the GATEWAY™ BP Clonase™ or LR Clonase™Enzyme Mix (Invitrogen). The system incorporates a negative selection marker (ccdB) that selects against vectors that have not undergone a recombination reaction, resulting in a high frequency of recovery of recombined plasmids.

1. The full length gene was PCR amplified using pfu polymerase forward (attB1: 5'GGGGACAAGTTTGTACAAAAAGCAGGCT3'+ GENE SEQUENCE) and reverse (attB2: 5' GGGACCACTTTGTACGAAAGCTGGGT+ GENE SEQUENCE) primers.
2. The amplification was checked by agarose gel electrophoresis for yield and product size.
3. The PCR product was precipitated by diluting the PCR reaction with 3 vol of TE and 2 vol of 30mM MgCl₂.
4. The precipitate was collected by centrifuging at 13,000 rpm for 10 min, supernatant was discarded and the pellet was dissolved in 10 µl TE.
5. BP reaction was set up by mixing 2 µl of BP clonase buffer, 2 µl of PCR product (150ng/ µl), 2ul of pDONR221 (150 ng/ul) and 2 µl TE.
6. The reaction was incubated at room temperature (25°C) for overnight. Next day 1 µl of proteinase K was added and incubated for 15 min at 37°C.
7. 2 µ of the reaction mix was used to transform the E.coli competent cells and plated on kanamycin plates.
8. Colonies were screened by sequencing and positive colony was selected for proceeding with the LR reaction.
9. LR reaction was set up by mixing 2 µl of LR colonase buffer, 2 µl (100-200 ng) pDONR 221 positive clone, 2 µl of vector pK7FWG2 and 2 µl of TE and the reaction was incubated for 1-16 hrs at room temperature
10. Next day 1 µl of proteinase K was added to the reaction mixture and incubated for 10 min at 37°C. Further 2-4 µl of the reaction mix was transformed to DH5α competent cells.
11. Colonies were screened by PCR for the presence of the insert. The positive colony was further transformed to Agrobacterium.
3.3.5.2 Agrobacterium transformation

3.3.5.2.1 Making competent Agrobacterium

1. Culture was initiated in 10 ml YEP media by inoculating a single colony of EHA 105 with appropriate antibiotics and grown overnight at 28 °C.
2. Next day 90 ml of YEP was inoculated with 0.5 ml of overnight grown culture and incubated at 28°C until the ABS600 reached 0.5 (the cells should be at early- to mid-log phase, it usually takes over night).
3. The flask was chilled on ice for 15-30 min and centrifuged for 15 min at 4°C 4000 x g. From this stage the cells should be kept cold throughout the preparation.
4. The supernatant was removed and the cells were resuspended in 30ml of 1mM HEPES pH 7.4. Then the cells were centrifuged again and the pellet was resuspended in 10ml of 1mM HEPES.
5. Finally the cells were centrifuged and resuspended in 2ml ice-cold 10% glycerol and dispensed in to microcentrifuge (45μl/tube) tubes. The tubes were snap frozen in liquid nitrogen and store at -80°C.

3.3.5.2.2 Transformation by Electroporation

Transformation was done by electroporation using Gene Pulser X Cell™ (Bio-Rad).

1. The electroporator cuvettes were chilled by keeping them in -20°C for 30 min. Then competent Agrobacterium cells were thawed on ice for 5 min.
2. To the thawed tube 50-100 ng of the plasmid to be transformed is added and gently tapped. Then the contents of the tube were transferred to the chilled cuvette.
3. The mixture of cells and the plasmid were brought down to the bottom by gently tapping the cuvette.
4. Then 0.1 mm cuvette was placed in the shock pod and electroporated using the preset protocol for Agrobacterium tumefaciens with Voltage of 2400, capacitance of 25 μF and resistance of 200 ohm’s.
5. After electroporation the cuvette was kept on ice for 2 minutes. To the cuvette 1 ml of YEP media without any antibiotics was added and all the contents were pipetted out to a fresh microcentrifuge tube.
6. The cells were incubated at 28°C for 2- 2 1/2 hours and then plated on YEP media with antibiotics. Further the plates were incubated at 28 °C for 2-3 days. The colonies obtained were patched on another plate and the transformation was confirmed by colony hybridization.

3.3.5.2.3 Colony hybridization

1. Colonies were patched on the plate containing selection media and allowed them to grow to optimum size.
2. After that, the patched petri-dishes were pre-cooled for at least 30 minutes at 4°C. The membrane was cut and bent in such a way that the resulting trough touched the centre of the petri-dish.

3. The membrane was released slowly to sit on the surface. The disc positions on the plate were marked at several positions using needle to ensure correct orientation of the colonies for subsequent analysis.

4. After 2 minutes the membrane was removed from the plate in one continuous movement using blunt ended forceps and placed colony side up on a 3mm Whatman paper.

5. An initial lysis step in which the membrane was placed colony side up on 10% SDS soaked 3MM Whatman paper for 5 minutes to liberate DNA from the colonies. Then the membrane was transferred to denaturation solution for 5 minutes and further to neutralization solution for 5 minutes, but care was taken to remove as much fluid as possible from the underside of the membrane by transferring briefly to dry 3MM paper between treatments.

6. Cell debris from the colony lifts was removed by vigorously washing the membrane disc in 2x SSC. Membrane was air dried and UV crosslinked (1200kJ/cm²).

3.3.5.3 *Agrobacterium*-based transient transformation

To shorten the time for gene functional analysis in fruits, transient expression of foreign genes is a valuable tool. We employed this tool to assess the role of mannosidase in ripening and/or softening. For transient transformation, we followed the method of Orzaez et al., 2006.

1. *Agrobacterium* cultures (3 mL) were grown overnight from individual colonies (Transformed with RNAi vector) at 28°C in YEP medium plus selective antibiotics, 200 μl of it was transferred to 50mL induction medium (0.5% beef extract, 0.1% yeast extract, 0.5% Peptone, 0.5% Sucrose, 2 mM MgSO₄, 20 mM acetosyringone, 10 mM MES, pH 5.6) plus antibiotics, and again grown overnight.

2. Next day, cultures were recovered by centrifugation, resuspended in infiltration medium (10 mM MgCl₂, 10 mM MES, 200 mM acetosyringone, pH 5.6; optical density 1.0) and incubated at room temperature with gentle agitation (20 rpm) for a minimum of 2 hrs.

3. Culture was collected with a syringe, and injected in the fruits as described below.

4. Tomato fruits (*Solanum lycopersicum* cv Pusa Ruby) at different stages of development were infiltrated using a 1-mL syringe. Needle was introduced 3 to 4 mm in depth into the fruit tissue through the stylar apex, and the infiltration solution was gently injected into the fruit.

5. The total volume of solution injected varied with the size of the fruit, with a maximum of 600 μl in mature green tomatoes. The progress of the process could be followed by a slight change in color in the infiltrated areas. Only completely infiltrated fruits were used in the experiments. Tomatoes at developmental stages beyond breaker did not infiltrate completely using this method and therefore were not included in the experiments.
3.3.5.3.1 Molecular analysis

3.3.5.3.1.1 Real-Time RT PCR

Real-Time RT PCR is a sensitive and powerful tool for analyzing RNA. The technique is very sensitive, rapid and specific, permitting analysis of gene expression from very small amounts of RNA (Gachon et al., 2004; Freeman et al., 1999). Real time differs from classical PCR by the measurement of the amplified PCR product at each cycle throughout the PCR reaction. In practice, a video camera records the light emitted by a fluorochrome incorporated into the newly synthesizes PCR product. Thus, real-time PCR allows the amplification to be followed in real-time during the exponential phase of the run, and thus allows the amount of starting material to be determined precisely. There are many detection systems like Taqman system, which uses a fluorescence resonance energy transfer (FRET) probe as a reporter system. A FRET probe is short oligonuclotide that is complementary to one of the strands. The probe contains a reporter and a quencher molecule at the 5' and 3' end of the probe, respectively. During the reaction as the Taq DNA polymerase enzyme replicates the new strand of DNA, the nuclease activity degrades the FRET probe at the 5' end, which is bound to template DNA strand. This degradation releases the reporter fluorochrome from its proximity to the quencher, resulting in fluorescence of the reporter. Accumulation of fluorescence reporter as a result of amplification of the target, can be detected by optical sensing units. This approach is an indirect way of the measuring the amount of DNA. The Molecular beacon technology is another direct approach that uses FRET-based fluorescent probes to bind the amplified DNA. In the unbound state, the quencher and reporter fluorochromes are maintained in close proximity via a hairpin loop designed into the sequence of the probe. Binding of the probe at the complementary strand separates the two fluorochromes, thereby alleviating the FRET interference and allowing the reporter to fluoresce (Walker, 2002). Another direct method which is commonly used is SYBR green, the fluorescent intercalating dye binds nonspecifically to the double stranded DNA and the accumulation is measured. This dye has the property of giving a specific fluorescent signal when bound to double-stranded DNA. Therefore fluorescence increases with the formation of PCR product.

In our work we used SYBR green to monitor the expression online with the light cycler 2.0 (Roche diagnostics) detection method. Gene specific primers were synthesized for tomato mannosidase as well as capsicum using the light cycler probe design software 2.0 and also respective actin primers were used as an endogenous control. The primers were having Tm of 60 °C and amplified a product of 150 bp. As a thumb rule, the amplicon size should be less than 700 bp for real time RT-PCR.

1. First step was to synthesize cDNA from RNA of the treatments or the stages which were under investigation, which was done by the protocol described in section (3.3.2.2) using adapter primer and Superscript II.

2. Then the cDNA was diluted 5 times with autoclaved MQ. The reaction was set up in laminar air flow without switching on the white light.
3. The PCR mix was prepared in the laminar air flow wearing powder free gloves. In a 1.5 ml microcentrifuge tube following components were added/reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
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<tbody>
<tr>
<td>Water, PCR-grade</td>
<td>9 µl</td>
</tr>
<tr>
<td>PCR Primer 10 mM</td>
<td>1+1 µl</td>
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<tr>
<td>Master mix (SYBR Green)</td>
<td>4 µl</td>
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4. The cocktail was mixed and centrifuged briefly. Then it was dispensed in to the precooled light cycler glass capillaries placed in light cycler centrifuge adapter.

5. Five µl of cDNA was added to each capillary and sealed by a cap using capping tool. The capillaries were carefully centrifuged at 2500 g for 5-10 sec and transferred to the Light Cycler sample carousel.

6. For each treatment three replicates of sample as well as the endogenous control were taken and mean was used for calculating the relative expression (RE) level. The RE level was calculated using $2^{-\Delta\Delta Ct}$ method.

3.3.5.3.1.2 Western analysis

Protein from agroinjected fruits (both blank vector injected as well as ihp-AMS injected) was extracted in 100 mM Tris-Cl pH 7.8 overnight and 50 µg of protein was precipitated with 20% TCA and resolved on 12.5% SDS-PAGE. Then transferred to nitrocellulose membrane and detected by anti-AMS antibodies using chemiluminiscence method as described in chapter II.

3.3.5.3.1.3 Isolation of Small interfering RNA, Blotting and Hybridization

Total RNA was isolated as described before in the section 3.3.4.1. For isolation of siRNA, procedure described by Dalmay et al., 2000 was followed.

1. The RNA after the precipitation with LiCl, was washed with 70% ethanol and the pellet was air dried.
2. The pellet was dissolved in 1ml of DEPC water and heated for 65°C for 5 min, and than placed on ice for 2 min.
3. To precipitate the high molecular weight RNA, polyethylene glycol (molecular weight 8000) was added to a final concentration of 5% and NaCl to a final concentration of 0.5M. After 30 minutes incubation on ice, the RNA was centrifuged at 13,000 rpm for 30 min.
4. The supernatant was precipitated with 3 volumes of ethanol and 1/10 volume of 3M Sodium acetate (pH 5.2) and the tube was placed at -20 °C overnight.
5. Next day the low molecular weight RNA was pelleted by centrifugation at 13,000 rpm for 10 min. The pellet was air dried and dissolved in 50 µl of DEPC water.
6. Then the siRNA was run on 15% Urea PAGE containing 0.5X TBE overnight. Next day the gel was transferred to nylon membrane using 0.5 X TBE for 1 hr at 100V constant.

7. The nylon membrane was UV cross-linked and pre-hybridized in 50% formamide, 7% SDS and 50mM Na₂HPO₄ and NaH₂PO₄ pH 7.2 at 40 °C.

8. After 4 hours of pre-hybridization the probe was added after denaturing for 5 min at 100°C. Hybridization was done for 14-16 hrs followed by washing.

3.3.5.3.1.3.1 Washing
Washing of the blot was done at room temperature with 2X SSC and 1% SDS for 5 min and further washed with 0.5 X SSC and 0.1% SDS for 5 – 10 min depending up on the count.

3.3.5.3.1.4 Small RNA isolation using mirVana Isolation kit
Total RNA was isolated as described in the section (3.3.4.1) and RNA was enriched for small RNAs using mirVana™ miRNA isolation kit (Ambion). This enrichment is accomplished by first immobilizing large RNAs on the filter with a relatively low ethanol concentration and collecting the flow-through containing mostly small RNA species.
1. To the total RNA 1/3 volume of 100% ethanol was added and mixed by vortexing or inverting the tube several times.
2. This mixture was loaded on to the filter cartridge and centrifuged for 15 sec at 10,000 rpm. Care was taken not to spin the cartridge harder, which may damage the filters.
3. The filtrate was collected in a fresh tube and to this 2/3 volume of 100% ethanol was added and mixed thoroughly.
4. This lysate/ethanol mixture was passed through a new filter by centrifuging at 10,000 for 15 sec and the flowthrough discarded.
5. 700 µl of miRNA wash solution 1 was added and centrifuged as in step 4, then 500 µl of wash solution 2/3 was applied and centrifuged. Additional spin of 1 min was given to remove residual fluid from the filter.
6. The filter was then transferred to fresh collection tube and to it 100 µl of pre-heated (95°C) elution solution or nuclease-free water was added to the center of the filter and centrifuged at 10,000 rpm for 30 sec. The eluate obtained contains small RNAs and stored at -20°C.

3.3.5.3.2 Texture analyzer
Texture analysis was done using TA XT-2 Plus, texture analyzer, Stable Microsystems, UK. Among many, compression is the simplest test of texture measurement. The sample is deformed and the extent of the deformation and/or the resistance offered by the sample is noted and used as an index of the texture of the food. In our case we used 75 mm compression plate to analyze the fruit texture. We performed two different kinds of compression, one 5 mm and the other 10 mm
compression on the whole fruit. Then the pericarp was cut into circular pieces and 2 mm compression analysis with a P/2 cylindrical probe was done to evaluate the texture of the pericarp.

1. Agroinjected as well as the \text{T}_0 fruits were analyzed for their texture. The agroinjected fruits were harvested after 7 days of injection and stored at RT for 25 days.

2. The texture analyzer was calibrated for its height before the compression test. The 75 mm cylindrical plate was fitted and its height was calibrated.

3. TA settings were adjusted according to the requirement and the trigger force was set to 5 g.

4. For each fruit three measurements of 5 mm compression, one measurement of 10 mm compression (because the fruit breaks after 10 mm compression) and 3 measurements of pericarp penetration were recorded and analyzed by unpaired t-test.

3.3.5.3.3 Microscopy
Microscopy was done at 100X magnification using Nikon 80i epiflouresent/phase contrast/Bright field microscope.

3.3.5.3.3.1 Toluidine Blue O and calcofluor staining
Toluidine Blue O is a basic dye which is used to stain the cell wall of plants tissue. We stained according to the procedure of O’Brien et al., 1964 with minor modifications.

1. The pericarp sections were frozen to -20 °C and sectioned on a sliding cryostated microtome. The samples were cut with 10-15 micron thickness and sections were dried on the slide.

2. An aqueous solution of 0.05% toluidine blue O in 0.1 M phosphate buffer (pH 6.8) was prepared and the slides were dipped in staining solution for a minute and destained in water.

3. The sections were mounted under the cover slip and all the liquid around the slide was removed and viewed under the microscope. The cell wall stained in reddish purple or red colour.

Calcofluor staining was done following the protocol of Parker (1984).

1. To examine the wall structure, the sectioned pericarp was immersed for 2 minutes in 0.1% calcofluor white, an optical brightener which is known to bind to some polysaccharides in the cell wall.

2. The sections were washed in autoclaved water and air dried. Then the sections were viewed in fluorescence microscope using appropriate filter.
3.3.5.3.4 Time lapse photography
The agroinjected fruits were harvested after 7 days of injection and stored at room temperature upto 45
days. The fruits were photographed every 10 days to observe the change in texture. The photography
was done with Canon G6 powershot with 4X zoom.

3.3.5.4 *Agrobacterium* based-Stable transformation
To known the gene function, we transformed the tomato with antisense, RNAi and overexpression
constructs. The procedure is described in detail below.

3.3.5.4.1 Developing antisense lines
Antisense lines were developed by transforming the tomato with *SLAMS* in antisense orientation driven
by CaMV 35 S promoter in pBI 121 Binary vector is described below.

3.3.5.4.2 Gene silencing by RNAi
RNAi lines were developed by transforming the tomato with RNAi constructs in pHANNIBAL vector
as described before. The transformation procedure is described below.

3.3.5.4.3 Over expression lines
Over expression lines were developed in tomato using the gateway binary vector PK7FWG2 as
described below.

3.3.5.5 Tomato Transformation
*Agrobacterium* mediated tomato transformation was done following the protocols of McCormik et al.,
1986 and Fillati et al., 1987 with few modification.

3.3.5.5.1 Seed Sterilization and germination.
1. Seeds were washed with autoclaved MQ for two times in the laminar.
2. After removing the water, the seeds were incubated for 11-12 min in 4% sodium hypo chlorite.
3. Seeds were then washed with autoclaved water for 10-12 times to remove all the traces of
sodium hypo chlorite and left overnight soaked in the same water.
4. Next day the seeds were placed in the germination media (1/2 MS pH set to 5.6-5.8) in the
vials
5. Then the vials were placed in the room temperature (25-28°C) for 5-8 days then shifted to
culture room once >50 % of seeds were germinated.
3.3.5.5.2 Preparation of cotyledons

1. Seedlings which were 9-11 (when the first true leaves are about to come) days old were taken for ex-plant cutting. Gently the tip and the petiole of the cotyledon were cut by rocking the scalpel blade to minimize tearing of the tissue, and only the middle portion was used as ex-plant for transformation.

2. The cut cotyledons were placed up right down on regeneration media (MS + zeatin pH set to 5.6-5.8).

3.3.5.5.3 Explant preparation and transformation

1. Two days before explant cutting, the Agrobacterium primary culture was initiated from single colony in 3 ml culture and grown for 2 days at 28 degrees.

2. On the day of ex-plant cutting, 50 ml YEP with appropriate antibiotics was inoculated using 100 μl of primary culture and grown overnight until the OD reached 0.7-0.8.

3. The culture was pelleted by centrifuging at 6000 rpm for 10 min at RT and the pellet was resuspended in 50 ml of MS media with 0.1μM acetosyringone.

4. The cotyledons were co-cultivated with resuspended culture in the petri plate for 30-45 min with gentle swirling.

5. Then the explants were blotted on Whatmann paper and placed right side up on the regeneration media.

6. The plates were kept at 28 degrees for 48 hrs and then the explants were transferred to 250 mg/l cefotaxime containing plates.

7. After 2 days the explants were transferred to the selection media containing 50 mg/l kanamycin along with 250 mg/l cefotaxime. After 2-3 weeks when the callus developed the drying ex-plant was excised from the callus.

8. Every 12-15 days the callus was placed on fresh regeneration media until the shoots appeared.

9. When the shoots were 2-3 cm long, they were cut and placed in rooting media (MS+IAA) until the roots developed.

10. When the roots developed completely, the plantlet was removed from the media and transferred to sterile soil in the pot.
3.3.5.6 Molecular analysis of the transformants

3.3.5.6.1 Isolation of Genomic DNA from Tomato

Genomic DNA was isolated as mentioned by Doyle and Doyle (1987) with some modifications.

1. Five gm tissue was frozen in liquid nitrogen, crushed using pestle and mortar, transferred to fresh SS-34 tube and 5-8 ml extraction buffer (2% CTAB, 1.4M NaCl, 20mM EDTA, pH 8.0, 100mM Tris-HCl, pH 8.0, 100mM β-ME) was added to the ground tissue.

2. Subsequently, the tubes were transferred to 60°C and incubated for 1 hr with intermittent shaking. After one hour the tubes were cooled to room temperature and to this, 5-8 ml of chloroform: isoamyl alcohol (24:1) was added and mixed gently and incubated for 1 hr followed by centrifugation at 10,000x g for 10 min at room temperature.

3. The upper aqueous phase was transferred to another SS-34 tube, and once again DNA was extracted with 5-8 ml of chloroform: isoamyl alcohol (24:1). To the final aqueous phase 0.6 volume of isopropanol was added for precipitating the genomic DNA and left overnight.

4. The genomic DNA was then washed thrice with 70% ethanol, dried in vacuum, dissolved in TE containing 10 mg/ml RNase and incubated at 37°C for 30 min. This was followed by extraction with phenol: chloroform: isoamyl alcohol (25:24:1) and the aqueous phase was transferred to a fresh tube.

5. Thereafter the genomic DNA was precipitated by adding equal volume of isopropanol. The pellet was obtained by centrifugation at 10,000x g for 20 min at 4°C and washed with 70% ethanol, air dried and dissolved in TE or nuclease free water.

3.3.5.6.2 PCR analysis

The putative transformants were checked for the presence of the vector by using intron specific primers.

1. Leaves from the putative transformants were harvested and frozen in liquid nitrogen. The leaves were crushed with micro pestle and the genomic DNA was isolated.

2. PCR was performed by using 50 ng genomic DNA of the putative transformants. The PCR reaction consisted of the following components

   - 10X PCR buffer 2.5 µl
   - 25 mM MgCl$_2$ 1.5 µl
   - 10 mM Left primer 1.0 µl (5'GTTAAGGTGATGTTAATTAGTATG 3')
   - 10 mM Right primer 1.0 µl (5'CAAATGTAAGATCAATGATAACAC 3')
   - Genomic DNA (50ng/µl) 1.0 µl

   Total volume made to 25 µl with Autoclaved water.

3. The PCR product was further resolved and analyzed on 0.8 % agarose gel with appropriate positive and the negative control.
3.3.5.6.3 Gene expression analysis
The putative transformants were analysed for reduction in the gene expression using real time RT-PCR. For this we used Step One RT PCR (Applied Biosystems) and analysed the result using comparative 2^ΔΔCt method. This reaction was set in 48 well plates taking vector transformed as the control. For each sample three replicates for the target and three for the endogenous control (Actin) were used.

1. The cDNA was prepared using the 3' RACE kit (Invitrogen) and diluted 5 times with autoclaved MQ.
2. RT-PCR cocktail was prepared one with Gene specific primers (Target) and the other with actin specific primers (Endogenous control) with each reaction containing 10 μl of Power cyber green, 6 μl of water, and one μl each of forward and reverse primers (10 μM).
3. The cocktail was dispensed in the 0.1 ml fast optical 48 well reaction plate and to each well 2 μl cDNA was added separately. Above the plate, 48 well optical adhesive film was firmly stuck with the help of applicator.
4. The plate was spun briefly in plate centrifuge and placed in the heating block of step One RT-PCR machine. Initial 10 min of denaturation was given followed by 40 cycles of denaturation (10 sec), annealing (10 sec) and extension (10 sec). After the reaction, melting peaks/curves were analysed to evaluate the specificity of the reaction.

3.3.6 Gene regulation

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<td>Mannosidase gene expression in the mutants</td>
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<td>.2</td>
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<td>.3</td>
<td>ACC treatment</td>
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</table>

3.3.6.1 Mannosidase gene expression in the mutants
Mannosidase gene expression was checked in most of the known ripening impaired mutants.

1. Flowers were tagged at anthesis and fruits were collected from mutants at different stages of ripening, corresponding to the wild type fruits.
2. The pericarp was then used for preparing cDNA and the expression was checked using real-time RT PCR. The expression was calculated using comparative ΔΔ Ct method and expressed in %, relative to the wild type.

3.3.6.2 Geraniol treatment
Tomato seedlings were given geraniol induction as described.

1. The seeds were surface sterilized and placed on MS medium with 0.6 % agar. Then they were kept in the normal day/night regime for two weeks.
2. Fifteen days old seedlings were uprooted slowly without wounding the roots and placed in the liquid media/water containing 10 mM geraniol in 20 % DMSO.
3. Samples were collected by freezing the seedlings at 0 min (Before inducing), 15th min, 30th min and 60th min of incubation in the geraniol containing media. The expression of the gene was analysed by real time RT-PCR, relative to the control (0 min).

3.3.6.3 ACC treatment

Tomato seedlings were treated with 1 mM ACC (Precursor of ethylene) similar to geraniol treatment. 15 days old seedlings were uprooted and placed in liquid MS media containing ACC. The samples were harvested at 0 hr, 2 hr and 12 hr of incubation and frozen in liquid nitrogen. Then the cDNA prepared from the samples were used to determine the expression of the gene, relative to the control (0 hr).

3.4 Results

<table>
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<tr>
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<td>8. Molecular analysis of the RNAi lines</td>
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<td>9. Geraniol induces mannosidase</td>
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<tr>
<td>10. Manlosidase is regulated by ( \text{rin} )</td>
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3.4.1 Constructions of cDNA expression library

Complementary DNA (cDNA) libraries represent the information encoded in the messenger RNA (mRNA) of a particular tissue or organism. RNA molecules are exceptionally labile and difficult to amplify in their natural form. For this reason, the information encoded by the RNA is converted into a stable DNA duplex (cDNA) and inserted into a self-replicating lambda vector. For this purpose we constructed cDNA expression library of capsicum and procured a pre-made library of tomato (Stratagene) to isolate the mannosidase clone from both the systems. Out of these, tomato has been classified as climacteric and capsicum in to non-climacteric based on the respiratory behavior and ethylene production during ripening. The presence of mannosidase activity in tomato and capsicum, belonging to \textit{solanaceae} family but distinctly differing in the ripening mechanism prompted us to isolate mannosidase from both the species.

The detailed flow chart of synthesis and processing of cDNA is depicted in the Figure 3.1A-B. We used the polyclonal antibodies produced against the enzyme as the probe to screen the expression library. For preparation of library we isolated 500 \( \mu \text{g} \) of RNA using TriPure reagent (Fig. 3.2A) and mRNA from PolyATract mRNA isolation system IV (Promega Corporation, Madison USA). After isolating the mRNA, the ratio of 260/280 was determined which was 1.95 indicating the purity of the mRNA. The mRNA was converted to cDNA and the size was determined by alkaline agarose gel (Fig. 89).
Figure 3.1 (A) Flow chart depicting synthesis and processing of cDNA for unidirectional cloning into Uni ZAP XR vector for construction of capsicum expression library. (B) Map of Uni ZAP XR insertion vector.
3.2B). The size of the cDNA after second strand synthesis was about 7-8 Kb. After cDNA fractionation using CL-2B gel filtration column and resolving on non denaturing PAGE, revealed the same (Fig. 3.2C). The fractions were pooled based on the size requirement. The cDNA was then quantitated on agar plate by spotting 0.5 µl of the cDNA (Fig. 3.2D). Based on the concentration three different ligations were set using three different pools of the cDNA. The ligation mix was packaged and the titer of the expression library was determined to be $1 \times 10^6$ pfu/ml. This primary library was further amplified, after amplification the titre was $1 \times 10^9$ pfu/ml and stored in aliquots. The average size of the capsicum library was 1.03 kb (Fig. 3.3A) and this was used for further work. The premade library was also titred and estimated to have $1 \times 10^9$-$1 \times 10^{11}$ pfu/ml. The average library size of the tomato was 1.3 Kb (Fig. 3.3B). These two libraries were screened with the polyclonal antibody of the mannosidase.

3.4.2 Degenerate primer design and cloning of mannosidase
Two approaches were simultaneously used to clone the mannosidase gene. First one was screening the expression library of capsicum and tomato using the polyclonal antibody. The second one was by polymerase chain reaction using degenerate primers. For the second approach, the LC-MS/MS data and the motifs identified by multiple alignments of the sequences from *Arabidopsis* and rice were used to design degenerate primers. The IUPAC ambiguity codes which were used during degenerate primer design are given in the Figure 3.3C. Out of the few primers one set of the degenerate primers (Left primer corresponding to the peptide QHVADDYAK 5' CAACATGKCTATGATATTAGCMA and right primer corresponding to the peptide SGAYVFRP 5'TGGRCGAAAMACATATGCTCCAGA) amplified a fragment of 0.7 Kb corresponding to mannosidase from capsicum and tomato (Fig. 3.4 A-B) using cDNA library stock as template in PCR. The peptides being conserved between the two same primers worked in both the system. The amplified fragments were cloned in pGEMT-Easy vector (Fig. 3.4C). The missing portions of 3' end were amplified using 3' RACE (Random Amplification of cDNA Ends) kit of invitrogen (Fig. 3.5 A). Both in tomato and capsicum 1.2 Kb fragment was amplified by 3' RACE (Fig. 3.5B-C). Further the 5' region was amplified using 5' RACE (Fig.3.6 A-D). The gene was made to full length by overlapping all the fragments and then end to end primers were designed to amplify the full length. Both in tomato (Fig. 3.7 A) and capsicum (Fig. 3.7 B) a fragment of ~ 3.1 Kb was amplified. Then the amplified fragments were cloned in pGEM-T Easy and checked by linearizing (Fig. 3.7C-D). The Blast-X analysis revealed that both the genes had homology to *Arabidopsis* mannosidase (Fig. 3.8) with E value of 0.

3.4.3 In-silico analysis
Using the ORF finder we identified the coding region of tomato to be 3090 bp long with 64 bp 5'UTR and 257 bp 3' UTR (Fig. 3.9A). The capsicum mannosidase coding region was 3093 bp long with 148 bp of 3' UTR and 236 bp of 5' UTR. The deduced protein from tomato was named as *SlAMS*
Figure 3.2 Construction of capsicum cDNA expression library. (A) RNA isolated by TriPure reagent and resolved on 1.2% formaldehyde agarose gel (B) Autoradiogram showing first strand and second strand synthesized using ZAP cDNA synthesis kit followed by resolution on alkaline agarose gel (C) cDNA fractionation on CL-2B gel filtration column and resolved them on 5% non-denaturing PAGE (D) Ethidium bromide plate assay to determine the concentration of cDNA.
Figure 3.3 (A) Agarose gel showing the average insert size of the fragments in the capsicum library. Randomly 10-12 plaques were picked up and amplified using vector specific primers. The average insert size was 1.03 Kb. (B) Average size of the tomato library was determined to be 1.3Kb. (C) Tables showing IUPAC ambiguity codes for all the amino acids which were used for designing degenerate primers.
Figure 3.4 PCR amplification of AMS using degenerate primers (A) from tomato library cDNA stock as the template. (B) Similar amplification in capsicum using the same degenerate primers. (C) Vector map of pGEM-T Easy used to clone the amplified PCR fragments.
Figure 3.5 (A) Flow diagram of 3’RACE procedure using oligo dT adapter primer which binds the poly A tail of the mRNA. Reverse transcriptase converts mRNA into ss cDNA. The RNA is then degraded by RNase H and UAP/AUAP primer with one gene specific primer (GSP) is used to amplify the target. 3’ RACE in (B) tomato and (C) capsicum amplified a fragment of 1.2 Kb and was resolved on 1% agarose gel.
Figure 3.6 (A) Mechanism of BD SMART™ cDNA synthesis. First strand synthesis is primed using a modified oligo(dT) primer. After reverse transcriptase reaches the end of the mRNA template, it adds several dC residues. The BD SMART IIA oligonucleotide anneals to the tail of the cDNA and serves as an extended template for BD powerscript reverse transcriptase. (B) 5' RACE in tomato and capsicum amplified a fragment of ~2.0 Kb. (C) and (D) Secondary amplification of primary PCR product from capsicum and tomato to confirm specificity of the amplification.
Figure 3.7 Full length amplification of α-mannosidase from both tomato (SlAMS) and capsicum (CaAMS) from cDNA prepared from respective RNA. The full length clone was 3.090 and 3.093 Kb in tomato and capsicum respectively. The amplicon was cloned in (B) pGEM-T Easy vector and (C) checked by linearizing, mannosidase cloned in pGEMT-Easy.
**Tomato Blast X**

**Reference:**

**Database:** All non-redundant GenBank CDS translations+PDB+SwissProt+PIR+PRF excluding environmental samples from WGS projects

5,371,785 sequences; 1,856,722,781 total letters

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**Figure 3.8** Blast X results of (A) SLAMS and (B) CaAMS sequences against non-redundant database. Result revealed a significant homology to the Arabidopsis mannosidase with E value of 0.
Tomato mannosidase

Length: 1029 aa
**Figure 3.9A** Tomato mannosidase ORF (open reading frame) predicted by ORF finder (NCBI). The largest ORF was 1029 amino acid long.
Cloning and Characterization of α-Mannosidase

(Solanum lycopersicum Alpha-mannosidase) which was 1029 amino acid long with calculated mass of 114 KD and the theoretical pl was 6.48. The deduced protein from capsicum was 1030 amino acid long (Fig. 3.9B) and was called CaAMS (Capsicum annum Alpha mannosidase) with theoretical pl of 6.28 and a calculated mass of 116.5 kD. In-silico prediction for protein subcellular localization revealed by TargetP, SignalP and Loc Tree servers indicated that the protein has a signal peptide for secretion (Fig. 3.10A). The hydropathy plot for tomato and capsicum mannosidase was determined by using TmPred server (Fig. 3.10B). The multiple alignments with known homologues from different plant species identified many conserved regions within the gene (Fig. 3.11). TMpred server predicted three transmembrane helices with N-terminus outside for tomato and two transmembrane helices for capsicum. The tomato α-mannosidase protein had 66% identity and 78% homology with Arabidopsis (Q8LPJ3) and 62% identity 79% homology with rice (ABG22500) mannosidase protein. Phylogenetic analysis (Fig. 3.12) revealed that it formed a close cluster with two Arabidopsis proteins (Q8LPJ3 and Q9FKW) and rice (EAZ18551.1) homologs. The modular organization of the deduced SlAMS protein sequence of tomato mannosidase (Fig. 3.13) revealed that the N-terminal, which corresponds to 5′ region of the gene, has "N-terminal glycosyl hydrolase domain" and C-terminal, corresponding to 3′ region of the gene, having "C-terminal glycosyl hyrolase domain ". Further, C-terminal domain also had part of the sequence similar to geraniol responsible factor15 (GRF15) (Fig. 3.13). GRF 15 was one of the clones which was upregulated when shoot primordia of Matricaria chamomilla was induced with geraniol (Ashida, 2002b). The middle region of the protein had glycosyl hydrolase middle domain with a structure consisting of three alpha helices, in an immunoglobulin/ albumin-binding domain-like fold. Like the SlAMS, CaAMS also had a similar kind of organization in the protein sequence (Fig. 3.14). Using the protein sequence, comparative homology modeling was done to predict the 3D structure for both the proteins. 3D JIGSAW server built a protein model using 1HTY (PDB entry) as the template. The template used was Golgi mannosidase (GMII) from Drosophila melanogaster and viewed with PyMol viewer (Fig. 3.15A-B). The accuracy of the query to the template was 95%. GMII from the Drosophila is a key enzyme in N-glycan processing which was considered as the target in the development of anticancer therapies. The crystal structure of GMII in the absence and presence of the anti-cancer agent swainsonine and the inhibitor deoxymannojirimycin reveals a novel protein fold with an active site zinc intricately involved both in the substrate specificity of the enzyme and directly in the catalytic mechanism (van de Elsen et al., 2001)

3.4.4 Expression analysis

We performed northern blot analysis to see the expression of SlAMS at different stages of ripening (Fig.3.16A). Transcript accumulated to maximum in the breaker stage followed by green, pink and minimum in the red stage. This result was also confirmed by real time RT-PCR (data not shown). Transcript accumulated to very low amount in leaf, root and stem (Data not shown). In case of capsicum (CaAMS) maximum transcript accumulated at stage 8 of ripening and was relatively constant.
Capsicum mannosidase

Length: 1030 aa

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194 ctggggctctggtggggaaagcgactttaggtgcttctcctcctcctcctc
S Q S I V K O L N V H L V P
244 cacgctgatgagttggtggtggaatactctgatgac
H S H D D V W L K T I D Q Y
292 tattggtcctttttatcaggggtcaatgtctggtgaaat
Y V G S H N S Q Q A C V E N
341 gcttgagctttttctgcagttttctgtggcatgatggaagcaggg
V L D S M V P A L A D K N R
381 aagttcacttttttttttatttatttttttttttttttttttatttttttattttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
Figure 3.9B Capsicum mannosidase ORF (open reading frame) predicted by ORF finder (NCBI). The largest ORF was 1030 amino acid long.
**Tomato TargetP 1.1**

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**Capsicum TargetP 1.1**

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**Figure 3.10A** *In-silico* prediction of subcellular localization and signal peptide in SIAMS and CaAMS. The prediction was done by using TargetP and SignalP servers against the plant & eukaryotic networks respectively.
Figure 3.10B Hydropathy plot of SIAMS and CaAMS predicted by TMpred server. For both the proteins the N-terminal was predicted to be exposed outside the cell and presence of three and two transmembrane helix for tomato and capsicum mannosidase, respectively.
**Figure 3.11** Multiple alignment of CaAMS and SlAMS with protein sequences of mannosidase from different plant species using Clustal W (EBI tool). Following were the plant species used in the alignment with their corresponding accession number.

- **NP 1969022**  
  *Arabidopsis thaliana*
- **NP 001031878**  
  *Arabidopsis thaliana*
- **NP189306**  
  *Arabidopsis thaliana*
- **NP 001067993.1**  
  *Oryza sativa (Japonica)*
- **ABE88173**  
  *Medicago truncatula*
Figure 3.12 Phylogenetic analysis of mannosidase sequences from other species. The tree was constructed using Mega 3.1 program using neighbourhood joining method.

Following are the sequences used in the phylogenetic analysis along with their corresponding accession numbers.

NP 1969022  Arabidopsis thaliana
NP 001031878  Arabidopsis thaliana
BAB11126.1  Arabidopsis thaliana
NP189306  Arabidopsis thaliana
NP001064140  Oryza sativa
AAL73068.1  Oryza sativa
EAY77673  Oryza sativa
EAY81127  Oryza sativa
EAZ18551.1  Oryza sativa (Japonica)
NP 001067993.1  Oryza sativa(Japonica)
ABG22500  Oryza sativa(Japonica)
ABG65906.1  Oryza sativa(Japonica)
NP 201416  Arabidopsis thaliana
ABG65905.1  Nematostella vectensis
Figure 3.13 Modular organization and sequence of the 1029 residue SIAMS protein deduced from SIAMS cDNA. The protein has three domains, N-terminal glycosyl hydrolase, middle glycosyl hydrolase domain and C-terminal glycosyl hydrolase domain as depicted in the cartoon. Peptide tags obtained by LC-MS/MS analysis are underlined. Dotted line shows sequence identical to McGRF 15 (Geraniol responsible factor 15). Rectangular box represent the predicted cleavage site between 24-25 position (SignalP).
Figure 3.14 Modular organization and sequence of the 1030 residue CaAMS protein deduced from CaAMS cDNA. The protein has three domains, N-terminal glycosyl hydrolase, middle glycosyl hydrolase domain and C-terminal glycosyl hydrolase domain as depicted in the cartoon. Peptide tags obtained by LC-MS/MS analysis are underlined. Dotted line shows sequence identical to McGRF 15 (Geraniol responsible factor 15). Rectangular box represent the predicted cleavage site between 24-25 position (SignalP).
Figure 3.15 Comparative homology modeling of SlAMS and CaAMS using 3D JIGSAW server and PyMOL viewer. The template used for building these structure was 1HTY (PDB) from Drosophila.
throughout the remaining stages. The pattern of transcript accumulation was very much similar to the protein level at different stages (Fig. 3.16B).

3.4.5 Transient suppression of alpha-mannosidase

Generation of stably transformed plants is a lengthy process. To determine the function of a gene, we tried to transiently suppress the endogenous gene expression using RNAi vector which produces double stranded RNA. Foreign gene expression using agroinjection has become common practice (Goodin et al., 2002 and Orzaez et al., 2006) and a handy tool for analyzing the gene function. Using this technique we silenced the endogene with pHANNIBAL vector (Wesley et al., 2001). As the regeneration and transformation of capsicum is not well established all the further experiments to functionally characterize the gene were performed in tomato using SLAMS. For this, 600 bp fragment of the SLAMS gene from either 5' or 3' region including respective UTR regions was sub cloned in sense and antisense orientation separated by an intron in pHANNIBAL vector which has a silencing efficiency of 90% (Fig. 3.17A). Further the NotI fragment from the pHANNIBAL vector was cloned in to binary vector, pART27 (Fig. 3.17A-D). This vector was able to produce inverted hairpin RNAs corresponding to the gene and was called as intron-spliced hpRNA (ihpRNA)(Fig. 3.17A-D). An overexpression vector (Fig. 3.18) with GFP fused at the C-terminal of the protein and an antisense vector in pBI 121 both driven by CaMV 35S promoter were prepared and agroinjected to validate the results (Fig. 3.19). We referred these construct as ihp-SLAMS (RNAi vector), Ovex-SLAMS (Over expression) and Anti-SLAMS (Antisense) in our future discussion. In order to mobilize these vectors in to the plant, they were transformed to Agrobacterium by electroporation and confirmed by colony hybridization (Fig. 3.20). The Agrobacterium was grown up to 1 OD and the cells were resuspended in infiltration media. These cells were injected into the pericarp of the fruit according to the method described. Each fruit was injected at 2-3 spots on the surface of the fruit near the stylar apex (Fig. 3.21) depending upon the size of the fruit so that infiltration media spreads to the maximum area of the fruit. Fruits could only be injected at mature green stage or at breaker stage. After a week the RNAi agroinjected fruits developed green sectors around the injected spot covering the maximum area around the fruit. The green sectors were more prominent and spread to a larger area in case of fruits agroinjected with ihp-AMS compared to control (Blank binary vector) (Fig. 3.21). The over expression vector injected fruits also developed green sectors at the site of injection and disappeared after few days, very much similar to the blank vector injected fruits. The Anti-SLAMS injected fruits performed marginally better than the control. To confirm suppression at molecular level, fruits were harvested after 2-4 days of injection and used to check for reduction in mRNA level corresponding to SLAMS by Real Time RT-PCR (Fig. 3.22). Real Time RT-PCR revealed that the mRNA level was reduced by 70% when injected 2 days before green stage and up to 50% when injected at green stage relative to the control (Fig. 3.23A). This reduction in mRNA level prompted us to look for the presence of small (21-23 mer) molecular weight RNAs (siRNA). We wanted to determine whether formation of small interfering
Figure 3.16 (A) Northern blot showing the expression of α-Mannosidase gene at different stages of ripening (G, Green, B, Breaker, P, Pink, R, Red Ripe) in tomato. (B) Real time RT-PCR analysis, using SYBR green, was done to determine the transcript level of mannosidase in various capsicum stages (Stage1-Stage8). Mannosidase transcript accumulated to highest level in breaker stage of tomato and stage 8 in capsicum. Due to non specific amplification in stage 3 (S3) of capsicum, the result of that stage was omitted during plotting of the graph.
Figure 3.17 Diagram depicting the steps in development of RNAi vector. (A) Vector map of pHANNIBAL. (B) Shows the closer view of the multiple cloning sites and the strategy used for cloning. (C) 0.8% agarose gel depicting products of different steps followed in cloning of pHANNIBAL. 2 is pHANNIBAL (HNBL) vector, 3 is HNBL digested with Xho I/Kpn I, 4 is first ligation, 5 is first ligation check, 6 is antisense fragment ligation, 7-8 ligation check of both (sense and antisense) the fragments, 9 is blank HNBL digested with Not I, 10 is HNBL with sense and antisense fragments digested with Not I, 11 is pART27 binary vector, 12 is pART27 digested with Not I, 13 is pART27 with ihp cassette digested with Not I, 14-15 digestion of pART 27 with Xho I/KpnI and Xba I/Cla I to release sense and antisense strands.
### Figure 3.18

Strategy followed for construction of AMS over expression construct (AMS-ovrex). (A) Full length gene was amplified with primers appended with attB1 and attB2 sites and resolved on 1.0 % agarose gel. (B) Vector map of pDONR221 in which the amplified gene was cloned. (C) This was further cloned in to destination vector pK7FWG2 with GFP fused at the C-terminal. (D) Colonies were screened for the presence of the fragment with gene specific primers. Lane 2-6 are positive clones compared with blank vector in lane 1.
Figure 3.19 Schematic diagram showing construction of antisense (Anti-AMS) construct

(A) Map of pBI 121 used for the antisense cloning
(B) Amplification of full length SLAMS with primers having Sac I and Xba I restriction sites appended to forward and reverse primers, respectively.
(C) Restriction digestion of pBI 121 with Xba I/Sac I to remove the GUS gene and confirming the cloning of SLAMS gene by digesting with the same enzymes.
(D-E) Confirming the orientation of the gene by restriction digestion.

The details of the enzymes used are given below:

Lane 1 and 4, 1 Kb ladder (Invitrogen)
Lane 7 and 8, 1 Kb ladder (Bangalore genei)
Lane 2 and 3, Amplification of full length with primers appended with restriction sites
Lane 5 pBI121 restricted with (Xba I/ Sac I) to release the GUS gene (1.8 Kb)
Lane 6 pBI-Anti SLAMS digested with Xba I/ Sac I to release the AMS gene (3.0 Kb)
Lane 8 Similar to lane 6
Lane 9 pBI-Anti SLAMS digested with EcoRI to release a fragment of 1.4 Kb, another fragment is due to the star activity of the enzyme.
Lane 11 pBI-Anti SLAMS and Lane 12 is Hind III restriction digestion releasing a fragment of 3.6 Kb
Figure 3.20 Colony hybridization to confirm transformation of *Agrobacterium* strain EHA 105 with (A-B) RNAi construct (C) Antisense construct (D) Over-expression construct. Vector transformed *Agrobacterium* was used as negative control. 5HM-1-3 and 3HM-1-3 is RNAi construct transformed, ANTI is antisense transformed, OVREX-1-3 are over expression vector transformed *Agrobacterium.*
Figure 3.21 Transient suppression of AMS in tomato. (A) Schematic representation of the construct used for transient silencing of the AMS. 5'or 3'region of the gene including the UTR was used under the transcriptional control of CaMV 35S promoter. Gene fragments were cloned in inverted orientation using the shown restriction sites, generating ihp repeats. (B) Fruits agroinjected with pHANNIBAL RNAi vector, targeting AMS gene. Blank binary vector and AMS-over expression vectors were used as control. The black /white arrows shows the injection mark on the fruit pericarp. Few fruits also developed cracks due to injection injury.
Figure 3.22 (A-B) Amplification curves obtained by amplification of target and reference during Real time RT-PCR analysis in Light cycler 2.0 (Roche). (C-D) The specificity of the amplification was checked by melting curves and melting peaks.
RNAs (siRNAs) was responsible for silencing of the endogenous gene. To better estimate the extent of AMS silencing, we prepared soluble small RNAs from green and breaker fruits of ihp-AMS and control fruits and performed northern blot analysis. We could observe small interfering (si) RNAs in the fruit pericarps which are the hallmark of RNAi. We could see both 23 as well as 21 nt siRNAs in the study (Fig. 3.23 B). Further the pericarp of the agro-injected fruits was used for immunoblot analysis to check the level of AMS protein. There was reduction in the AMS protein in ihp-AMS compared to the vector injected fruits with varying degrees from 20-90% (Fig. 3.23C). The injected fruits were harvested and stored at room temperature for 30 days and analyzed for their texture.

3.4.6 Fruit texture analysis

Fruit texture was quantified in terms of pericarp and flesh firmness. The fruit after agro-injection were harvested and subjected to firmness measurement using a Texture Analyzer (TA-XT Plus, Stable Microsystems, UK) fitted with a probe. We analyzed fruit and pulp firmness of agroinjected fruits by compression analysis (Fig. 3.24A). As ripening is not a uniform process we measured the fruit firmness by compressing the fruits through 5mm at different planes of the fruit (2-3 compressions/fruit). Then the same fruit was compressed through 10 mm with stylar end touching the plate of the texture analyzer. After 10 mm compression, most of the fruits collapsed, hence it gave the maximum force (in kg) a fruit can withstand. 

In another set, circular piece of 1.5cm diameter fruit pericarp discs were excised from the agroinjected fruit and the firmness of the endocarp was measured using the P/2 cylindrical probe by placing the discs upside down with skin touching the plate of the texture analyzer. In all the cases, ihp-AMS agro injected fruits and their pericarps were significantly firmer and were able to withstand more force than the blank vector injected fruits (Fig. 3.24B-D). In case of 5 mm compression, the fruits were two times firmer/stronger than their counterpart; 10mm compression and pericarp compression revealed that they were 1.6 times firmer than the control.

In order to determine the shelf life, the agroinjected fruits after the harvest were stored at RT (23°C) and they were photographed every 10 days (Fig. 3.25) to track their deterioration. It was observed that the ihp-AMS injected fruits retained their texture and firmness compared to control which started shrinking and loosing their texture after 20 days. After 30 days we stained the transverse pericarp sections with Toludine blue and Calcofluor which binds to some polysaccharides in the cell wall (Fig.3.26). Although there was no much difference in the cell wall architecture, but we could notice the ihp-SIAMS agroinjected fruits depicted much compact organization with more carbohydrates deposited in the cell wall.

3.4.7 Stable transformation

Development of plant genetic transformation, which is an efficient technique essential for the exploitation of molecular biology in both fundamental and applied research, offers new tools for molecular breeding. Here, in Tomato, we have used the Agrobacterium strain EHA105 for
Figure 3.23 Molecular analysis of agroinjected fruits (A) SYBR-Green RT-PCR analysis of α-mannosidase gene expression in fruit peel after agroinjection with RNAi vector corresponding to mannosidase gene compared to the fruits agroinjected with blank binary vector. (B) Autoradiogram showing siRNA signal of 23 and 21nt in agroinjected fruits. (C) Immunoblot analysis of agroinjected fruits. 50 µg of protein was loaded on SDS-PAGE and transferred to nitrocellulose membrane and hybridized with anti-AMS antibody. In 3.23 B, lane 1-3 represent fruits agroinjected with ihp-AMS and lane 4-5 are blank vector (Control) injected fruits. M is marker. In 3.23 C lane 1-6 and 7-8 are agroinjected samples, C is vector injected (control) fruit and +ve represents purified mannosidase.
Figure 3.24 Texture analysis of Agroinjected fruits. (A) A typical graph obtained by fruit compression/penetration analysis using TA-XT2 plus texture analyzer. The significance was calculated by t-test. (B) 5 mm Fruit compression using 75 mm plate. \( P \leq 0.0001 \) and \( n=43 \) for ihp-AMS and \( n=27 \) for control. (C) 10 mm Fruit compression using 75 mm plate. \( P \leq 0.0005 \) and \( n=19 \) for ihp-AMS and \( n=13 \) for control. (D) Peel compression using P/2 cylindrical probe. \( P \leq 0.0028 \) and \( n=24 \) for ihp AMS and \( n=16 \) for control.
Figure 3.25 Time lapse photography showing representative agroinjected fruits. Fruits were stored at room temperature for 45 days. Control fruits represent the fruits agroinjected by blank vector and ihp- SLAMS fruits represent transiently silenced fruits.
Figure 3.26 Light microscopy images of agroinjected fruit cuticles to study the cell wall after 30 days. Light microscopy images with Toluidine Blue O (A, B) or with Calcofluor (C, D) of Control (Blank vector) as compared to Toluidine Blue O (E, F) and Calcofluor (G, H) of ihp-AMS injected fruits.
transformation (Hood et al., 1993). This strain contains L, L-succinamopine Ti plasmid which makes it hyper virulent and therefore high frequency of transformation was obtained. The *Agrobacterium* infection has already been used to transfer important traits like shelf life, fungus resistance, insect and virus resistance in tomato. The transfer of T-DNA from *agrobacterium* to plants is dependent on a virulence functions outside the T-region of the Ti plasmid. T-DNA is delineated by 25 bp inverted repeat sequence called right border and left border. In order to develop stable RNAi lines we established a regeneration and transformation system in tomato. Tomato being a model crop, for taking up of development and ripening related studies, we choose this crop. The most efficient DNA delivery system for introducing foreign genes into tomato is the *Agrobactrium* mediated transformation of cotyledonary leaves (McCormick, 1991). The cotyledonary leaves were used as ex-plants for regeneration of tomato. The tomato regeneration and transformation protocol was established in our lab (Kesarwani et al., 2000). The protocol uses tobacco feeder cells for the transformation process and takes longer time. Therefore, we adopted a modified protocol of McCormik et al., 1986 and Fillati et al., 1987 which takes comparatively less time. The regeneration of tomato is depicted in the Figure 3.27A-V. After rooting the plants were hardened and transferred to pots in the green house (Fig. 3.28G-H). The silenced fruits were photographed at different stages of ripening (Fig. 3.28A-E). For determining the shelf life as well as the texture of the fruits, they were harvested at green stages and kept at room temperature for 30 days. To evaluate the extent of silencing, we performed molecular analysis of the transgenics.

### 3.4.8 Molecular analysis of the RNAi lines

To confirm the integration of the silencing cassette, genomic DNA was isolated from the putative transgenic plants and PCR analysis was done with intron specific primers. The transgenic plants amplified 0.7 Kb intron specific DNA (Fig. 3.29A-B). Further, the extent of suppression was determined by real time RT-PCR. The results of RT-PCR revealed that the suppression of endogenous mannosidase ranged from 60% - 98.5%. The RNAi lines 3HM-4, 3HM-5, 3HM-6, 3HM-7 and 3HM-8, showed suppression more than 90% as compared to wild type (vector transformed) (Fig. 3.29C). The fruits from the transgenics were harvested and stored up to 30 days and then their texture was analysed (Fig. 3.29D-E) by compression analysis with 75 mm compression plate. The compression test revealed that the mannosidase silenced fruits were significantly firmer than the control (vector transformed). The 5 mm compression revealed that the transgenics were 2- 2.7 times firmer than the control with 3HM-4, 3HM-5, 3HM-6 and 3HM-7 showing the best texture. 10 mm compression analysis revealed that fruits were 1-1.8 times firmer than the vector transformed.

### 3.4.9 Geraniol induces mannosidase

Plants respond to geraniol, which induces apoptosis-like cell death. The expression of Glutathione S-transferase (GST) was up regulated after treating cultured cells of *Glycine max* with 5 mM Geraniol
Figure 3.27 Transformation and regeneration of tomato to produce transgenic lines. (A-S) Represents different stages of regeneration, from ex-plant to the rooted plant. (T-V) Shows root initiation and development.
Figure 3.28 Transfer of T₀ generation RNAi plants to green house (A-E) SLAMS silenced fruits photographed at different stages of ripening (A-B) Green, (C) Breaker (D) Pink (E) Red (F) flower (G-H) potted RNAi lines in green house.
Figure 3.29 Molecular analysis of AMS RNAi (T₀) lines (A) Genomic DNA isolated from leaves of T₀ plants. (B) PCR amplification using intron specific primers. Lane 1-10 represent the RNAi lines whose (A) genomic DNA was isolated and (B) checked by PCR analysis. Lane 11 is the plasmid with intron (+ve control) and lane 12 is PCR analysis of wild type (negative control).
Figure 3.29 Molecular analysis of RNAi lines. (C) Real time RT-PCR analysis of T₀ fruits. Flowers were tagged at anthesis and cDNA prepared from breaker stage fruits was used. (D) Texture analysis of T₀ fruits with 5 mm (E) and 10 mm compression. Data are means ± SE (n=15). The significance was calculated by t-test with P value < 0.005.
and it is a kind of geraniol responsible factor (GRF) (Ashida et al., 2002a). Further, in *Matricaria chamomilla* cultured shoot primoridia geraniol induced *McEREBP1, McWRKY1, McGRF3, McGRF15, McGRF18* and *McGRF20* (Ashida et al., 2002b). Geraniol an acyclic dietary monoterpene, has *in-vitro* and *in-vivo* antitumor activity against various cancer cell lines and considered as new class of agents for cancer chemoprevention (Camesecchi et al., 2001). It was also reported that geraniol interferes with the membrane functions of *Candida albicans* and *Saccharomyces cervisiae* (Tsuchiya, 2001). The geraniol is also known to induce senescence and death of plant cells.

Due to the presence of sequence similar to geraniol responsible factor 15 (*McGRF15*) on C-terminal of SIAMS protein as well as CaAMS, we checked the effect of geraniol on expression of SIAMS. As geraniol is not soluble in water, 20% DMSO was used to feed the plants (Hendry and Jones, 1984). For this fifteen days old tomato seedlings grown in MS were taken and placed in 10 mM geraniol and another set of plants were placed in 20% DMSO as a control. Samples were collected at 15 min, 30 min and 60 min (Fig. 3.30A). Using real time RT-PCR, relative expression was checked with respect to 0 hr (control). We found that SIAMS was up-regulated up to 3 fold after 30 min of treatment. And after 1 hr the gene was down regulated compared to the control plants (grown in 20% DMSO) (Fig 3.30B). DMSO had no effect on seedlings as well as the expression of the SIAMS. Using the $2^{-\Delta \Delta C_T}$ method, the data is presented as the fold change in gene expression normalized to an endogenous reference gene and relative to the untreated control.

### 3.4.10 Mannosidase is regulated by *rin*

To gain molecular perspective on gene regulation we checked the expression of mannosidase in already known ripening impaired mutants like *rin*, nor and Nr fruits (Fig. 3.31B) and found that the transcript level was 90% reduced in *rin*, 30% reduced in Nr and nor expression level was comparable to the wild type (Fig. 3.31A). The *rin* fruit do not soften, produce only basal level of ethylene, do not accumulate carotenoid pigment (Giovannoni et al., 1989) and moreover have greatly reduced levels of alpha-mannosidase, relative to the wild type. This gave us the clue to further analyze the pleiotropic *rin* mutant. We investigated whether geraniol can induce AMS in *rin* mutant. We fed the *rin* seedlings with 10% geraniol and collected samples at 15, 30 and 60 min. Relative gene expression analysis revealed that SIAMS transcript was up regulated up to 6 fold after 30 min (Fig. 3.32) i.e. double the expression seen in wild type seedlings. This experiment made very clear that SIAMS is induced by geraniol.

Ripening-inhibitor (*rin*) carries a mutation in a gene encoding a MADS-box transcription factor (Vrebalov et al., 2002). Further *rin* mutant is also deficient in autocatalytic ethylene production but can sense ethylene from external source. Ethylene being another potent modulator of fruit ripening and best studied in tomato, we took help of ACC, precursor of ethylene, to induce the SIAMS in the seedlings. Ethylene was able to induce AMS in *rin* as well as wild type (pusa ruby) (Fig. 3.33A-B) giving a hint of the gene being controlled by ethylene.
Figure 3.30 Real time RT-PCR analysis using SYBR Green in Pusa Ruby to check its inducibility to geraniol. (A) Fifteen days old seedlings were fed with geraniol for 15, 30 and 60 min and RNA isolated from these were reverse transcribed. (A) The seedlings in 10 mM geraniol lost vigor and viability after 60 min of incubation. (B) The inducibility was indicated as compared to untreated (0 hr) seedlings. Data are means ± SE (n = 4).
Figure 3.31 (A) RT-PCR analysis to show relative expression of AMS in ripening impaired mutants rin, nor and Nr as compared to Ailsa Craig and Pusa Ruby. Data are mean ± SE (n=3) (B) Pictures of fruits from various ripening impaired mutants and wild type.
Figure 3.32 Real time RT-PCR analysis done in rin mutant seedlings using SYBR green. Geraniol inducibility in rin mutant was analyzed after feeding them with 10 mM geraniol in 20% DMSO. Data are mean ± SE (n=4). Black histogram are rin seedlings fed with geraniol and grey histogram are rin seedlings fed with 20% DMSO.
Figure 3.33 (A-B) Inducibility of SLAMS in response to ACC as revealed by real time RT-PCR. (A) Pusa Ruby and (B) rin seedlings were grown in MS media containing 1mM ACC up to 12 hr. Data are means ± SE (n = 4). Grey histograms represent the control and Black histogram represent the treatment.
3.5 Discussion

Here, we describe for the first time the cloning and characterization of alpha-mannosidase, member of glycosyl hydrolase family 38 of CAZy family from tomato and capsicum. One of the reasons to take up this work was that plants are known to contain far more carbohydrate-active enzyme (CAZyme) encoding genes than any other organism sequenced to date (Coutinho et al., 2003) and among them glycosyl hydrolases form the major chunk (Jamet et al., 2006). Although many processes have been assigned to glycosyl hydrolase family genes, very few genes have been experimentally proved. Moreover out of different glycosidases, mannosidase showed very high activity in the tomato and capsicum which prompted us to take up this work. Degenerate primers based on the LC MS/MS data were used to clone the gene. The screening approach failed due to enormous background obtained due to polyclonal antibodies. But the same library stock was used as template for PCR amplification using degenerate primers. The deduced amino acid sequence revealed to have significant homology to glycosyl hydrolase family 38 and was predicted to be a secretary protein. The transcript level was highest at breaker stage and further reduced up to red stage, very much similar to the protein level as detected by immunoblot analysis. In silico analysis like Clustal W and Phylogenetic analysis revealed its maximum similarity to Q8LPJ3 and Q9FKW from Arabidopsis and EAZI8551 and ABG22500 from Oryza, for which no functional characterization has been done, yet.

Prolonging the desirable texture during ripening is the key to prolonging the shelf-life of the fruit (Chapple and Carpita, 1998). Therefore, in order to examine our proposed hypothesis, of its role in fruit ripening/softening, transient silencing of the mannosidase gene was done in the fruit with pHANNIBAL vector which can produce ihp (intron spliced) fragment with a silencing efficiency up to 90%. Agroinjection suppressed the gene up to 70% very much similar to TRV-VIGS system (Da-Qi-Fu et al., 2005) which suppressed the LeACS-2 gene up to 73% compared to control fruit (vector alone) in another study. We found that surface agroinjection in one fruit did not transfer to another fruit on par with TRV vectors (Da-Qi-Fu et al., 2005). Our study establishes that non viral vectors can also give the similar silencing effect by producing siRNA molecules. In our study we found two classes of siRNA molecules, 23nt and 21nt as reported earlier (Hamilton et al., 2002) each with a different role. As a result of silencing there was marked difference on the texture of the fruit. The AMS silenced fruits were 1.6-2 times firmer than the control (Blank vector) and the force they were able to withstand after 30 days was similar to that of the pink stage fruit (Data not shown). We stored the agroinjected fruits for six weeks at room temperature. The mannosidase silenced fruits stayed longer without any deterioration and without loosing the texture. This may be due to intact cell wall polysaccharides which are broken down to a lesser extent in silenced lines as visualized by toluidine blue O and calcoflour staining, which are known cell wall binding dyes (O’Brien et al., 1964 and Parker, 1984). The encouraging results in the agroinjection prompted us to produce stable RNAi lines for mannosidase. The stable RNAi lines had no phenotypic change and the fruits looked normal. The difference was visible only when they were stored for 20 days at room temperature. The T₀ fruits from
the silenced lines were 2-2.7 times firmer than the empty vector transformed lines, which was quite
evident from its texture. One more observation was made for T1 seeds, they were much bolder (bigger
in the area) than the control this may be because of its role in seed development. The silencing of the
gene may produce lot of pleotropic effect which needs to be carefully accounted and explained.

Changes in the mechanical properties of primary cell walls ('loosening' and 'tightening') are
evoked by auxins, gibberellins, ethylene and other hormones. These changes play a major role in
governing the rate and pattern of plant growth and development. But data available on AMS in plants
is very scanty. In order to identify the regulation of the gene we took help of the ripening impaired
tomato mutants rin, nor and Nr which are known to be affected in many aspects of fruit ripening. Out
of all the mutants, rin inhibited AMS gene transcription up to 90% relative to the wild type. rin fruit
synthesize basal levels of ethylene throughout development unlike the wild type and is also impaired in
autocatalytic ethylene production (Lincoln and Fischer, 1988). However exposure of rin seedlings to
ACC, precursor of ethylene, alleviates AMS gene expression. ACC also induced AMS gene in wild
type up to an extent and further repressed the gene. Considering these results we hypothesized
that AMS gene is regulated by changes in ethylene concentration and when the level of ethylene reaches a
threshold, autoinhibition represses the gene. Presence of sequence identical to Geraniol Responsible
Factor 15 (GRF15) at the 3' end of the gene prompted us to look for the geraniol inducibility. Geraniol,
an acyclic dietary monoterpene induced the AMS gene in wild type and to a greater extent in case of
mutant rin, indicates that it has role to play in development and ripening. Geraniol is known to induce
programmed cell death/senescence by DNA fragmentation (Ashida et al., 2002a). This was further
proved from our experiment where 1 hr of incubation in 10 mM geraniol resulted in loss of vigor and
viability, leading to death of the seedlings (Fig. 3.30A). Geraniol was able to induce AMS in wild type
and to a higher extent in rin mutant. To discuss this result we assumed that the mutant might be having
low amount of basal geraniol in the system compared to wild type which is therefore not sufficient to
induce the SIAMS gene. External application of geraniol to rin, induces it to a much higher extent
compared to wild type which already has higher amount of basal geraniol. One of the possibilities is
that the rin mutation may be inhibiting biosynthesis of geraniol and external application is sufficient to
activate the unknown geraniol receptor which will in turn pass the signal to the transcription factors to
activate the mannosidase gene. The observation that ACC, precursor of ethylene can induce SIAMS
gene in the rin mutant as well as the wild type made clear that AMS is ethylene regulated gene.
Although, ethylene synthesis is inhibited in rin mutant, it can sense the external ethylene and
upregulate the genes. Therefore, upon ACC treatment, AMS gene was expressed which may be either
regulated by ETR4 or Nr. The role of ETR4 in AMS gene expression needs to be confirmed. But
involvement of Nr can not be excluded as the expression of AMS gene in Nr background suppressed
the AMS gene up to 30% compared to the wild type (Fig. 3.31 A). Further the signal may be
transduced to the transcription factors and control the gene expression. Unlike the geraniol, ACC
induced alpha-mannosidase to a same level in mutant as well as in the wild type. This may be due to
autoinhibition of the ethylene after a threshold level. Therefore, we hypothesize that \textit{SlAMS} is regulated by ethylene as well as geraniol independently or synergistically. All the observations were put together to build a possible pathway for \textit{SlAMS} gene regulation (Fig. 3.34). But cloning of promoter and in depth analysis will further give a better insight in to its regulation. And also geraniol content at different stages of fruit ripening will further clarify the results.

Taking all the results together we can confidently say that \textit{\alpha}-mannosidase is involved in ripening associated softening but it is not sole determinant of this process. In today’s world of global distribution, the control of fruit ripening is of strategic importance (Causier et al., 2002). Therefore \textit{\alpha} mannosidase offers potential applications for bioengineering of cell walls, either to manipulate the growth and texture of plants or to modify the structure and physical properties of cell wall or shelf life improvement. However, this is a tip of iceberg: a big effort should be made to identify and discover the function of many more glycosyl hyrolases and various cell wall polysaccharide degrading enzymes which are participating in the cell wall degradation. One more question needs to be answered, unlike tomatoes, \textit{Arabidopsis} and \textit{Oryza}, which posses alpha-mannosidase homologues, are not fleshy and undergo a maturation process ending in senescence, dehydration and dehiscence. Functional analysis of AMS in such systems will lead to additional insights regarding maturation of different fruit types and its diverse roles. Finally, this work identifies a possible new candidate, Geraniol, as a signaling molecule in the plants which may trigger many more developmental processes.
Figure 3.34 Pathway depicting the possible gene regulation of SLAMS in response to ACC and geraniol. Red box □ indicates the pathway block or non functional pathway. Green box ■ indicates functional pathway. IP is interacting proteins, ERF is Ethylene responsive factor, EREBP is Ethylene responsive element binding protein, AMS is Alpha mannosidase.