Cloning and Characterization of AmA1 Gene Architecture
2. Cloning and Characterization of AmA1 Gene Architecture

2.1 Introduction

Amaranthus hypochondriacus is one of few non-graminaceous plants which produce significant amounts of edible cereal grains. It is also a pseudocereal and falls in the class of grain amaranth and it grows vigorously under adverse conditions. It is tolerant to drought, heat and many pests; adapt readily to new environment. AmA1, a 35 kDa protein was found to be a seed specific protein with high and balanced amino acid composition for a nutritional protein. The cDNA clone of AmA1 was isolated using affinity-purified AmA1 antibodies, cloned and sequenced (Raina and Datta, 1992). Studies from other plant systems have shown that storage protein gene expression is regulated by the combined influence of various cis-acting DNA sequences along with several trans-acting factors. Thus, it was interesting to know if similar control mechanisms exist in amaranth as well.

To study the regulation of this gene and to understand its role as seed specific protein, it was an objective to isolate the gene with its regulatory elements. As most of the seed storage protein genes have been reported to occur as a multigene family, it was of interest to know the copy number of AmA1 as well as the genome size of Amaranthus. Since, the cDNA clone was already reported, it is convenient to use the cDNA insert as a probe to screen a genomic library from Amaranthus. The phage replacement vector facilitates the cloning of large size insert. The advantages of using a λGEM12 XhoI half site arms as a cloning vehicle are:

1. It facilitates cloning of foreign DNA in the size range of 14-20 kb.
2. Partially filled in half site arms in the absence of insert prevents self ligation of vector molecules. This provides an enrichment for recombinant genomes which can be packaged efficiently.
3. The poly linker has SaeI, NotI, BamHI, EcoRI, XhoI and Xbal. Partially filled in XhoI arms can accept exogenous DNA fragments prepared by Sau3A1 partial digestion and end-filled. The partial digest provides fragments with random cleavage sites such that overlapping fragments are represented in the library.
4. The full length insert can be excised cleanly from vector arms using the NotI sites.
5. The λGEM arms with partially filled-in XhoI sites in conjunction with partially filled-in Sau3A1 digested genomic DNA eliminates the need of size-fractionation of genomic fragments since the partial fill-in vector prevents insert to insert ligation. The only ligation products possible are single copies of genomic inserts with the appropriate arms.
6. Since, no size fractionation of genomic DNA is required only with 10μg DNA a library can be obtained.
7. This vector has dual opposed T7 and SP6 promoters.
8. Asymmetric Sfil restriction sites flanking the cloning promoter region.
9. The central stuffer regions of the λGEM11 and 12 vectors contain no pBR322 sequences. Thus, the few non-recombinant plaques that do appear (1 in 10^5) will not hybridize with pBR322 derived probes.

The regulation and expression of this gene can be studied once the nucleotide sequence is available. To achieve this, the full length clone was isolated from the genomic library using 1.12 kb probe and sequenced. Comparison of the deduced amino acid sequence with that of other storage proteins and also the upstream sequence search would reflect any cis-elements, enhancers, silencers or trans-acting factor binding sites in the sequence for storage proteins. The cloned gene will eventually be used to map all promoter elements.

Seed storage proteins are expressed during seed maturation to high levels in restricted seed tissues such as cotyledons and embryos of dicots or endosperm of monocots. Their corresponding mRNAs are accumulated temporarily at the middle to late phases of seed development (Goldberg et al., 1989). Spatial and temporal expression of these genes make them excellent model systems for the study of molecular mechanisms underlying differentially regulated gene expression in plants. Like the situation in a number of highly regulated plant genes, cis-acting elements in the 5' upstream region regulate expression of these genes with trans-acting nuclear factors. The cis-elements which interact with trans-factors have been found in the 5' flanking regions of various seed protein genes such as helianthinin, phaseolin, conglycinin, legumin, zein, hordein, glutelin etc.

Since AmA1 transcripts is reported to be present only in seed at a particular phase of seed maturation (Raina and Datta, 1992), it was of interest to map cis-elements responsible for seed specific expression which could in turn bind with trans-acting nuclear factors like other class of storage protein genes. The ability of a protein to bind DNA can be detected by any one of the following methods: (a) nitrocellulose filter binding assay, (b) gel mobility shift assay, (c) exonucleaseIII assay, and (d) DNaseI protection assay. Gel mobility shift assays as described by Fried and Crothers (1981) was used to detect the ability of any seed specific trans-acting elements to bind cis-sequence. The advantages of gel mobility shift assays over the other methods are: (i) the assay is very sensitive as it provides information about the number complexes (factors) involved in binding, (ii) the specificity of the complex formed can be confirmed by competition experiment, and (iii) size of probe (target) can vary from short oligos upto 1 kb fragments, although large fragments would require further narrowing down of the binding site. We have used two regions of AmA1 promoter each of which has homology with cis-elements of other storage protein as targets for gel mobility shift assays.

2.2 Materials and Methods

1. All % shown are on a W/V basis (unless mentioned otherwise).
2. All solutions and media were made in Mili RO and Mili Q H2O.
3. Whenever needed the solutions and media were sterilized by autoclaving at 15lb/sq inch for 20 min or filter sterilized.
4. Chemicals used were of Analytical or molecular biology grade and the source mentioned wherever necessary.
5. Restriction enzymes and DNA modifying enzymes were obtained from NEB, BM, Promega. Buffers provided with the enzymes were used and digestions were carried out at the temperature recommended by the manufacturer.

2.2.1 Plasmids and Strains used:

- **Escherichia coli**
  - DH5α, LE392, KW 251, HB101
- **Bacteriophage**
  - λGEM12 (c1857)
- **Plasmids**
  - High copy no.
    - pBSIIkS+, pUC19, pTZ18U
  - Low copy no.
    - pBIN19
- **Plant Materials**
  - *Amaranthus hypochondriacus* var. annapurna.

2.2.2 Media and Solutions

- **LB Medium**
  - 1% tryptone, 1% NaCl, 0.5% Yeast Extract, pH adjusted to 7.5 with NaOH (Gibco).
- **LB agar**
  - LB with 1.5% agar (Gibco).
- **Top agar**
  - 1% Bactotryptone, 0.5% Yeast extract, 0.5% NaCl, 0.25% MgSO₄ (pH 7.0) with 0.7% agar.
- **SM Buffer**
  - 0.58% NaCl, 0.2% MgSO₄, 7H₂O, 50mM Tris-HCl (pH 7.5), 0.01% gelatin.
- **Media with Ampicillin**
  - LB with 50μg/μl ampicillin and LB-agar with 75μg/ml ampicillin.
- **LB Medium with Maltose and MgSO₄**
  - LB medium was supplemented with 0.2% maltose and 10% MgSO₄.
- **TB**
  - 1.2% Bactotryptone, 2.4% Yeast extract, 0.4% (v/v) glycerol.
- **10X phosphate Buffer**
  - 2.31g KH₂PO₄ and 12.54g K₂HPO₄ in 100ml H₂O.
- **2 X L**
  - 2% Bactotryptone, 1% YE, 0.1% NaCl, 0.2% glucose.
- **Trituration Buffer**
  - 100mM CaCl₂, 70mM MgCl₂, 40mM NaOAC (pH 5.5).
- **1XCTAB Buffer**
  - 50 mM Tris-HCl, pH 8.0, 0.7M NaCl, 10mM EDTA pH 8.0, 1% CTAB, 20mM 2-mercaptoethanol.
- **2XCTAB Extraction Buffer**
  - Double the concentrations of the constituents of 1XCTAB extraction buffer.
- **10% CTAB**
  - 10% (w/v) CTAB with 0.7M NaCl.
- **1X CTAB Precipitation Buffer**
  - 50mM Tris-HCl (pH 8.0), 10mM EDTA(pH8.0), 1% CTAB.
- **High-salt TE Buffer**
  - 10 mM Tris-HCl (pH 8.0), 1mM EDTA (pH8.0), 1M NaCl.
- **TE(pH 8.0)**
  - 10mM Tris-HCl (pH 8.0), 1mM EDTA (pH8.0).
- **EB**
  - 10mg/ml ethilium bromide.
20XSSC: 175.3g NaCl and 88.2g of Trisodium citrate was dissolved in 800ml H2O. The pH was adjusted to 7.0 by adding 100μl of HCl (50%) and volume was made upto 1 litre and sterilized by autoclaving.

3m NaOAc (pH 5.2).

7.5M NH4OAc (pH 7.5) and 5M NH4OAc.

RNase A: Prepared as a 10mg/ml stock as described in Maniatis et al., (1982).

Phenol: Glaxo laboratories Exel AR grade was redistilled at 180°C and stored frozen at -20°C in small parts.

Phenol:Chloroform:IAA: 30% ficoll 400, 60mM EDTA (pH 8.0), 0.6% SDS, 0.06% Bromophenol Blue.

6XEndoR: Prepared as a 10mg/ml stock as described in Maniatis et al., (1982).

Storage of Phage Stock: Phage liquid Lysate was stored with a drop of chloroform at 4°C after filtering.

Storage of bacterial strains: For long term storage, overnight grown bacterial cultures were stored as glycerol stocks. Bacterial cultures were grown overnight & sterile glycerol was added to a final conc. of 15% and stored at -80°C.


Phenol:Chloroform:IAA: 25 parts of TE saturated phenol, 24 parts of chloroform, 1 part of Isoamylalcohol

2.2.3 Isolation of High Molecular Weight Genomic DNA from *Amaranthus hypochondriacus* var. annapurna

Genomic DNA from *A. hypochondriacus* was isolated by the method of Rogers and Bendich (1994) with a few modifications.

Steps in the Procedure

1. Approximately 25g mature seeds were ground to a fine powder in pre-chilled mortar using liquid-N2 (2L) in cold room at 4°C.

2. The seed meal was transferred to a 250ml conical flask. To this, 1 volume (i.e.25ml) of hot (65°C) 2XCTAB buffer was added and maintained in a 65°C water bath. Then 7 Vol. (175ml) of 1 X CTAB buffer (preheated at 65°C) was added. The mixture was incubated at 65°C for 30 min with occasional swirling at every 5 min.

3. An equal volume (200ml) of chloroform:IAA (24:1) was added and mixed thoroughly to form an emulsion.

4. The phases were separated by centrifugation at 13000g for 10 min at room temperature (RT). (The aqueous phase should be of equal volume to that of debris; if not 1XCTAB buffer can be added to equalize this volume).

5. To the aqueous phase (200ml), 0.1 vol. (20ml) of 10% CTAB solution was added and mixed gently.
6. Another chloroform:isoamylalcohol (24:1) extraction was done by adding an equal vol. (220ml) of Chloroform:IAA. Phases were separated by centrifugation at 13,000g for 10 min at RT.

7. Aqueous phase was precipitated by addition of equal volume of CTAB precipitation buffer by gentle mixing and incubated at RT for 30 min.

8. Pellet was recovered by centrifugation at 2000g for 5 min. (NB : Centrifugation at higher speed or more time should be avoided because tight pellet is difficult to rehydrate).

9. Resulted pellet was rehydrated in 5ml of high salt TE buffer.

10. DNA was reprecipitated by addition of 2 vol. of cold absolute ethanol at -20°C for 30 min.

11. DNA Pellet was recovered at 6000g, for 10 min in 4°C.

12. The pellet was washed with 80% ethanol and dried in Speed vac.

13. The pellet was dissolved in 9ml TE (pH 8.0) and incubated at 55°C water bath for rapid dissolution.

14. While incubating 9.7g cesium chloride was added and the tube was kept at 37°C until DNA and CsCl goes to the solution. This was then left on ice for 30 min. Centrifuged at 6000g for 10 min to discard any particulate matter.

15. To the supernatant (11ml) 0.5ml of ethidium bromide (10mg/ml) was added and incubated on ice for 30 min. Centrifuged at 6000g for 10 min at 4°C. This pellet would contain RNA, protein and carbohydrate.

16. Supernatant was centrifuged at 55,000rpm for 22hr at 20°C using TV-865 rotor in Combiplus ultracentrifuge.

17. DNA bands were seen under hand UV-illuminator and recovered by using 1ml sterile tip (end was cut to avoid shearing of DNA).

**Removal of Ethidium Bromide and Cesium Chloride:**

1. To the DNA solution an equal volume of 1-butanol saturated with 20XSCC was added and mixed gently. After the separation of two phases, upper organic phase containing ethidium bromide was removed.

2. The extraction was repeated for 6-7 times until both the organic and aqueous phase became colourless.

3. The ethidium bromide free DNA solution was then transferred to a fresh tube and diluted 3 times with sterile MQ H2O and DNA was precipitated by two volume of cold absolute ethanol at -20°C, overnight.

4. DNA pellet was recovered by centrifugation at 6000g for 10 min at 4°C.

5. Pellet was washed with 80% ethanol, dried in Speed vac.

6. DNA pellet was dissolved in 0.4ml TE (pH8.0) and reprecipitated with 0.1vol. of 3M NaOAc (pH 5.2) and 2 vol. of absolute ethanol in eppendorf tube at -20°C.

7. DNA pellet was recovered by centrifugation, 80% alcohol wash was given, dried under vacuum and dissolved finally in 100μl TE (pH 8.0).
8. The concentration and purity was estimated by A260/A280 ratio as well as analysing on 0.4% agarose gel electrophoresis.

2.2.4 Agarose Gel Electrophoresis

Agarose gel electrophoresis of DNA was done as described in Maniatis et al., (1982). Routinely 1XTAE buffer was used for gel as well as tank buffer. Ethidium bromide was added to a final concentration of 0.5μg/ml both in the gel and in the running buffer. DNA was quantitated by comparison with known amount of intact λDNA at different concentration on a 0.4% agarose gel supported by a 1% agarose gel base.

2.2.5 Genomic Southern and Genome Complexity

To find out the copy number of AmAl Gene in Amaranthus hypochondriacus, genomic southern was done using complete cDNA insert as probe.

Genomic DNA Digestions: Amaranthus hypochondriacus genomic DNA isolated in section 2.2.3 was taken for each digestion.

1. 2.5μg of genomic DNA was digested in 20μl reaction volume at standard reaction condition for each enzyme. All enzymes used were from NEB. Enzymes used were BamHI, ClaI, EcoRI, HaeIII, HindIII, NcoI, PvuII, ScaI, SphI and XbaI. All the reactions were incubated at 37°C for overnight (16 hr).
2. From each digestion, an aliquote of 4μl (i.e. 500ng) was analysed on a 0.7% agarose gel to check the extent of completion of digestions.
3. Rest of the 16μl digest (2μg) was loaded on 14cm x 12cm 0.7% agarose gel containing 0.5μg/ml ethidium bromide along with the λ-HindIII digest and pUC19-HinfI digest as DNA molecular weight marker.
4. Gel was run with 1XTAE buffer at 40volt constant at the beginning; as all the DNA digest entered the gel voltage was increased upto 80.
5. After the dye (BPB) reached two third of the gel, run was stopped and gel was visualized on UV-transilluminator and photograph was taken by using Kodak 667 polaroid film along with a fluorescent scale.

Transfer of Target DNA onto Membrane:

1. The gel was rinsed with MQ H2O just after the run.
2. Depurination of the DNA within the gel was done by dipping the gel in 200ml 0.25N HCl for 15-30 min in a baking dish with mild shaking at 50rpm on gyratory platform shaker.
3. The gel was rinsed in MQ H2O to complete the depurination process.
4. DNA was denatured by soaking the gel in 200ml of 1.5M NaCl and 0.5M NaOH for another 30 min at similar conditions.
5. The gel was neutralized by immersing in 200ml of 1.5M NaCl and 0.5M Tris-HCl (pH 8.0) for 30 min.
6. It was then briefly equilibrated in transfer buffer, 10XSSC.
7. Duralone-UV membrane and 3mm whatman paper was also prewetted by floating on 10XSSC transfer buffer.
8. DNA was transferred onto Duralon UV membrane by the pressure blotting method, using PosiBlot pressure blotter (Stratagene) at 75mm Hg pressure for 1.5 hr.

Fixation of Target DNA onto Membrane:
1. After the transfer was complete the gel wells were marked on membrane with a needle.
2. The membrane was put into fresh 10XSSC transfer buffer, gently rubbed off with soaked cotton to remove any agarose gel residue.
3. Membrane was placed on a dry 3MM whatman paper and DNA was fixed by UV crosslinking at 1200 j/cm for 30 sec. Using stratalinker UV crosslinker (stratagene) and dried at RT for overnight (12-16 hr).

Prehybridization of Membrane: The membrane was prehybridized in 50% deionized formamide, 10% dextran sulphate, 1% SDS, 900mM NaCl at 42°C for 6-8 hr in hybridization oven with 0.035ml of prehybridization solution per cm² of the membrane in a hybridization bottle.

Hybridization: 1.12 kb EcoRI fragment of cDNA clone was used as probe. The probe was made by random primer labelling method and purified over spun column as mentioned in section 2.2.7.2. Total purified count was 9.3 x 10⁵ cpm/60μl. In the same prehybridization solution 9.3x10⁵ cpm probe was mixed along with 200μg/ml sheared salmon sperm DNA after denaturation at boiling water bath for 10 min followed by quick chilling for 5 min. Hybridization was carried out for 24 hr at 42°C.

Post Hybridization Washing:
1. Blot was washed with 1XSSC and 0.1% SDS for 15min at RT.
2. High stringency washing was done thrice using 0.1XSSC and 0.1% SDS at 60°C for 15 min each.
3. After washing, the membrane was briefly blotted on dry filter paper and sealed in a plastic bag.

Autoradiography: Membrane was exposed with kodak X-Omat film with two intensifying screen at -80°C for 6 hr.

2.2.5.1 Estimation of Genome Size

Genome size and minimum complexity of genome of *Amaranthus hypochondriacus* was determined by quantitative method.
1μg of pAmA1.3 cDNA clone was double digested with Sphi and BamHI along with 5μg of amaranth genomic DNA.

(a) First, 5μg genomic DNA and 1μg pAmA1.3 DNA was digested with Sphi in a total volume of 50μl and 20μl respectively at 37°C for 6 hr.
(b) Both the digestions were heat inactivated at 65°C for 20 min and DNAs were precipitated with 2 vol. of ethanol at -80°C for 1 hr.
(c) DNA pellet for both reactions were suspended in MQ H2O and digested with BamHI in a total reaction volume of 20μl for overnight (16 hr) at 37°C.
(d) pAmA1.3 digestion was serially diluted in the following way:
The Sphi-BamHI insert (0.621 kb) is about 16% of total pAmA1.3 size (3.869 kb). So, in 1μg pAmA1.3 digest, insert amount was 160.75ng/20μl digest i.e. 8.03ng/μl.
1. 1 μl of AmA1.3 digest + 998 μl 1XER = 1000μl (8pg/μl)
2. 25μl mix from Tube no. 1 + 75μl 1XER = 100μl (2pg/μl)
f) pAmA1.3 digests 2pg, 4pg, 6pg, 8pg, 10pg, 12pg, 18pg, and the 5μg of genomic DNA digest were loaded along with 1kb ladder as DNA molecular weight marker in a 0.7% agarose gel.
(g) Gel was ran at 80 volt constant for 4 hr, then was visualized under UV-transilluminator and trace was taken as later reference.

Transfer of Target DNA onto Membrane: Gel was transferred onto GeneScreen Plus nylon membrane as per DuPont GeneScreen Plus transfer and detection protocols.

Hybridization: 0.9 kb BamHI restriction fragment of pSB2 (described in section 3.3.7.1) was used to make probe by random primer labelling using NEbloc kit as per manufacturers protocol. The probe was purified through Sephadex G-50 column. Prewashing, prehybridization and hybridization was done according to GenescreenTM Plus protocol at 42°C with formamide. Probe concentration was 2.8 x 10^5 cpm/ml of hybridization solution and total volume was 6ml with 200μg/ml sheared salmon sperm DNA. Washing of the membrane was also done according to GeneScreen Plus membrane protocol.

Autoradiography: Blot was exposed with Kodak X-Omat film with 2 intensifying screens for 24hr at -80°C.

Densitometric Scan: Densitometric scan of the autoradiogram was done by using densitometer to find out the quantity of DNA in 0.621 kb insert of genomic DNA.

2.2.6 Construction of Genomic Library

Genomic library was made as per Promega protocol as follows:
2.2.6.1 Optimization of Sau3A1 Partial Digest of Genomic DNA

In order to establish the optimum enzyme concentration to generate a certain size range of fragments (15-23 kb), the following small scale reactions were performed:

**Solutions required:** Sau3A1 10X buffer - 100mM Tris-HCl (pH 7.5), 1M NaCl, 70mM MgCl2
Sau3A1 dilution buffer - 1X Sau3A1 10X Buffer, 0.1mg/ml acetylated BSA.

1. Serial dilutions of Sau3A1 (NEB, 4u/μl) were made in different tubes mentioned below on ice.

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Preparation</th>
<th>Final Enzyme Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.</td>
<td>1μl of Sau3A1 + 9μl dilution buffer</td>
<td>4u/10μl</td>
</tr>
<tr>
<td>B.</td>
<td>5μl of A + 5μl dilution buffer</td>
<td>2u/10μl</td>
</tr>
<tr>
<td>C.</td>
<td>5μl of B + 5μl dilution buffer</td>
<td>1u/10μl</td>
</tr>
<tr>
<td>D.</td>
<td>5μl of C + 20μl dilution buffer</td>
<td>0.5u/25μl</td>
</tr>
</tbody>
</table>

2. After the enzyme dilutions were made, small scale digestion reactions were set up in separate tubes as follows:

First, assay buffer was made for 3 reactions having 1μg DNA/reaction.

<table>
<thead>
<tr>
<th>Assay Buffer</th>
<th>Final Enzyme Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA (660ng/μl)</td>
<td>4.5μl (3μg)</td>
</tr>
<tr>
<td>10XSau3A1 buffer</td>
<td>15.0μl</td>
</tr>
<tr>
<td>10mg/ml BSA</td>
<td>1.5μl</td>
</tr>
<tr>
<td>H2O</td>
<td>154.0μl</td>
</tr>
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<td>135.0μl</td>
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</table>

Four tubes were taken numbered 1 to 3. In each tube 45μl assay buffer was taken having 1μg of DNA.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>1</td>
<td>45μl (1μg DNA) assay buffer + 5μl of tube A</td>
<td>2u/μg</td>
</tr>
<tr>
<td>2</td>
<td>45μl (1μg DNA) assay buffer + 5μl of tube B</td>
<td>1u/μg</td>
</tr>
<tr>
<td>3</td>
<td>45μl (1μg DNA) assay buffer + 5μl of tube C</td>
<td>0.5u/μg</td>
</tr>
<tr>
<td>4</td>
<td>45μl (1μg DNA) assay buffer + 5μl of tube D</td>
<td>0.1u/μg</td>
</tr>
</tbody>
</table>

3. Incubated all the tubes after mixing at 38°C for 30 min.
4. The reactions were terminated by addition of 10μl of 6X EndoR gel loading buffer.
5. The samples were analysed on 0.4% agarose gel with a 1% support. Electrophoresis was carried out at 2V/cm. The gel was photographed and the amount of enzyme needed to produce the maximum intensity of fluorescence in the desired size range was determined.

2.2.6.2 Large Scale Preparation

Using the optimized conditions determined in small scale standardization, the large scale reactions were carried out.

1. For large scale preparation of partially digested Sau3A1 genomic DNA, enzymes concentrations of 4u and 3u were chosen to set up digests of 10µg DNA in a reaction volume of 500µl respectively to yield fragments in the size range of 8-23 kb.
2. The Digests were extracted twice with phenol:Chloroform and finally with chloroform:IAA.
3. The agarose phase containing the DNA fragments were precipitated with 0.5 volume of 7.5M ammonium-acetate followed by 2 vol. of ethanol at -20°C for overnight.
4. The precipitate was washed with 70% ethanol and finally dissolved in 20µl TE (pH 8.0), thus DNA conc. was 0.5µg/µl.
5. 1µg/2µl from each digestion was analysed on 0.4% agarose gel along with uncut amaranth genomic DNA and λ-HindIII as molecular size marker.

2.2.6.3 Partial Fill-in Reaction for Cloning into XhoI Half-Site Arms

To clone the Sau3A1 digested fragments into XhoI half-site arms, the partial fill-in reaction was carried out.

Solutions required:

- Klenow 10X buffer (NEB)
- Klenow fragment (NEB), 5u/µl
- 20mM dATP and 20mM dGTP

Steps in the Procedure

1. Partial fill-in mix for two reactions was prepared as follows:
   - 10XKlenow buffer -- 20µl
   - 20mM dATP -- 10µl
   - 20mM dGTP -- 10µl
   - Klenow enzyme -- 2µl (10u)
   - MQ H2O -- 138µl
   - 180µl

90µl partial fill-in mix was dispensed in each tube.

2. 10µl (5µg) of partially digested DNAs were mixed with 90µl of partial fill-in mix and incubated at 37°C for 1/2 an hr.
3. Extracted twice with 1 vol. of TE saturated phenol:chloroform, vortexed for 1 min and centrifuged at 12,000g for 5 min to separate the phases.
4. Aqueous phase was extracted with Chloroform:IAA once as in step 3.
5. The aqueous phase was precipitated with 0.5 vol. of 7.5M ammonium acetate (pH 7.0) and 2 vol. of chilled ethanol at -80°C for overnight.
6. The DNA pellets were recovered by centrifugation at 10,000g at 4°C for 15 min.
7. Pellets were washed with 70% ethanol and vacuum dried and then dissolved in 10μl of MQ H2O each.
8. 1μl of each sample was estimated spectrophotometrically. A260/A280 ratio was 1.8 and DNA conc. in both the cases were 0.5μg/μl.

2.2.6.4 Ligation

For ligation, 0.5μg in 1 μl of partially filled-in Sau3A1 digests of 0.3μ/μg and 0.4μ/μg DNA's were taken. 0.5μg of partially filled-in λ–GEM12 XhoI half site arms (Promega) was taken as vector per ligation reaction.

Steps in the Procedure
1. 0.5μg of inserts and 0.5μg of vector were mixed in eppendorf tube on ice in a volume of 2μl.
2. Tubes were preheated at 45°C for 5 min and chilled quickly on ice.
3. Then 0.5μl of 10Xligase buffer was added along with 1 μL of NEB ligase in each tube and the volume was made up to 5μl.
4. Ligation was performed at 4°C in a multitemp for 16 hr.

2.2.6.5 Packaging of Ligated DNAs

Packaging was done by using the Gigapack II packaging extract from Stratagene.

1. 125 ng/1.25 μl of ligation mix was packaged in vitro using Gigapack packaging extracts at 22°C for 2 hr.
2. 0.125 ml of SM buffer was added followed by 5μl of chloroform. This primary library was stored at 4°C.

2.2.6.6 Titration of the Genomic Library

Preparation of plating cells: Host cells used - KW251

1. KW251 cells were streaked from glycerol stocks onto LB medium containing 15μg/ml tetracyclin; incubated at 37°C for overnight.
2. Single colony was inoculated in 10ml LB supplemented with 0.2% maltose and 10% MgSO4, and allowed to grow at 37°C at 200 rpm until A600 reached 0.5. Tetracyclin in
Preculture was omitted to avoid the formation of Mg\(^{+2}\) tetracyclin complex (Manniatis \textit{et al.}, 1982).

3. Grown preculture was stored at 4°C till use (NB: Plating bacteria should not be used after 48 hr of growth).

2.2.6.7 Plating and Estimation of Titer by Spotting

1. Phage serial dilutions of 10\(^{-1}\), 10\(^{-2}\), 10\(^{-3}\) of original library were made using SM-buffer.
2. 0.3 ml of plating cell culture was mixed with 3 ml molten top-agar (maintained at 48°C) and poured onto LB-agar plate supplemented with tetracyclin.
3. 5 μl of each dilutions along with 5 μl of original primary library were spotted onto these plates and incubated at 37°C for 6-8 hr till the plaques appeared as cleared areas against a lawn of bacterial cells.
4. From the number of plaque at a given dilution, the titer of the library was estimated.

2.2.7 Screening of Primary Library

1. Based on the titer from spotting method the whole library was plated onto 3 big plates (137 mm diameter) and 4 small plates (82 mm diameter).
2. For smaller plates 300 μl of KW251 plating cells at log phase (A\(_{600}\)=0.5) were mixed with 125 μl of phage library. For 137 mm plates 900 μl of same plating cells were mixed with 250 μl of phage library.
3. Phages and cells were mixed by brief vortexing and allowed to adsorb at 37°C for 15 min.
4. Molten top agar (3 ml for 82 mm plate and 9 ml for 137 mm plate) was maintained at 48°C before plating. The phage-cell mixes were mixed after incubated with required amount of top-agar, vortexed briefly and poured quickly on LB plates on a leveled surface. Top agar was allowed to harden and then the plates were incubated at 37°C.
5. As and when the plaques appeared, plates were removed from 37°C and stored at 4°C at least for 2-4 hr before plaque lifting. This helps in preventing the top agar from sticking to the membranes.

2.2.7.1 Plaque lifting and Membrane Treatment

1. For lifting the plaques, prelabelled duralon UV-membrane discs (Stratagene) were used. The membranes were handled as per the manufacturer's instructions.
2. The membranes were placed on the plaque side of the agar plate without trapping air bubbles for 2 min. Three asymmetric orientation marks were made by piercing the membrane and agar with a sterile needle. The plate was held up to a light and on the underside of the plate the position of the needle holes were marked.
3. The membranes were treated with 0.75 ml of denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 2 min at RT with the plaque side up such that the solution does not run over the plaque side.
4. Membranes were neutralized with 0.75mL of neutralization solution (1.5M NaCl and 0.5M Tris-HCl pH 8.0) for 5 min at RT in the same way as in step 3.

5. The membranes were then treated with 2XSSC and 0.2M Tris-HCl pH 7.5 for 5 min at RT.

6. The excess buffer from membrane discs were blotted on filter paper and the DNA on damp membrane was UV-crosslinked using Stratlinker UV cross-linker for 30 sec at 1200 joules/cm.

**Preparation of Dot Blot:** To get a positive control for hybridization, one dot blot was made using pAmA1.3 plasmid DNA at different concentrations starting from 10pg to 10ng along with λDNA (50ng) as negative control.

**Steps in the Procedure**
1. One strip of Duralon UV membrane was presoaked in 2XSSC and air-dried.
2. DNA samples to be spotted were denatured by boiling for 5 min and chilled quickly before spotting.
3. Membrane was air-dried before any further steps.
4. The membrane was denatured in 1.5M NaCl and 0.5M NaOH for 2 min at RT.
5. The membrane was neutralized in 1.5M NaCl and 0.5M Tris-HCl (pH 8.0) for 5 min at RT.
6. Then the membrane was treated with 2XSSC and 0.2M Tris-HCl (pH 7.5) for another 5 min at RT.
7. The excess buffer from the membrane was blotted on filter paper and the DNA on damp membrane was UV-crosslinked as described above.

2.2.7.2 Preparation of 1.2kb Probe

(a) Isolation of insert DNA: The plasmid, pTZ18U (USB) containing 1.2kb full length cDNA of the gene AmA1 (Raina, A. and Datta, A., 1992) was used to isolate the DNA fragment.

1. 5.5μg of pAmA 1.3 was digested with 20u of EcoRI in a total volume of 50μL with 1XECORI unique buffer at 37°C for 4 hr.
2. The digest was resolved on 0.7% agarose gel along with DNA size marker (λHind-III and pUC19-Hinfl digests).
3. Expected 1.2kb EcoRI fragment of cDNA was cut out from the gel with a sterile blade and the insert was purified using Qiaex DNA gel extraction kit (Qiagen) as per manufacturer’s instructions.
4. DNA was finally eluted in 20μL TE (pH 8.0) and quantitated again by agarose gel electrophoresis against known quantity of pUC-Hinfl digest.
5. Insert DNA concentration was 100ng/μL.

(b) Labelling of the Insert DNA by Random Primer Labelling Method: Labelling was done according to Feinberg and Vogelstein (1984).
Solutions required:
- 4XRHP buffer
- Nuclease free BSA (Pharmacia)
- dNTP mix (-dCTP) (Pharmacia)
- α 32 PdCTP (BARC, India)
- Klenow enzyme -5u/μl (NEB)

1. 50ng/0.5μl of insert DNA in a total vol. of 23μl was denatured in a boiling water bath for 5 min, followed by quick chilling on ice.
2. Following labelling mix was made on ice:
   - 4XRHP buffer: 12.5μl
   - BSA: 2.0μl
   - dNTP (-dCTP): 2.0μl
   - α 32 PdCTP: 7.5μl (50μCi)
   - Klenow: 1.0μl (5u)
   - Total: 25.0μl
3. Labelling reaction was then mixed with denature DNA solution and this total 50μl of labelling reaction was incubated overnight at RT.
4. The specific activity of the probe was 2.34x10^8 cpm/μg DNA.
   The % incorporation was 66%. Count was taken as per Berger, (1987).
5. The probe was purified on a 1ml sephadex G-25 column saturated in TE (pH 8.0), as per Sambrook et al., (1989).
6. Purified total count was 3.9x10^7 cpm/60μl volume.

2.2.7.3 Hybridization with Labelled Probe

Hybridization and washing of the filters were carried out as described in Stratagene Duralon-UV™ membrane instruction manual.

1. Filters as well as the dot blot was prewashed to remove the cell debris at 42°C in a waterbath in 5XSSC, 0.5% SDS and 1mM EDTA for 2hr with gentle agitation.
2. After prewashing step all the membranes with the plaque side up was gently swapped with a cotton soaked in 2XSSC.
3. The prehybridization, hybridization and washing were done in hybridization bottle inside hybridization oven (Robbins Scientific).
4. Prehybridization was performed in a solution containing 50% deionized formamide, 10% dextran sulphate, 1% SDS, 900mM NaCl at 42°C for 6hr.
5. In case of hybridization the probe was denatured along with 200ng/ml sheared salmon sperm DNA in a water bath for 10min, chilled on ice. Probe was added to a final concentration of 0.78 x 10^6 cpm/ml.
6. Hybridization was carried out at 42°C for 16 hr.
7. The probe was then poured off and stored at -20°C and the filters were washed with 1XSSC, 0.1%SDS at RT for 10 min followed by twice with 0.1XSSC and 0.1% SDS at 42°C for 15 min. The filters were finally washed once in 0.1XSSC and 0.1% SDS at 65°C for 15 min.

8. Filters were exposed wet in sealed plastic bags to kodak X-Omat AR films at -70°C with intensifying screens for 18 hr.

9. X-ray film was developed by using IPC kodak developer and fixer by standard method.

2.2.7.4 Picking up the Positive Signals

1. To orient the filters, the film was lined up and asymmetric marks were numbered on the film.

2. Then the films were oriented on the LB agar plate containing the primary library by using these asymmetric marks. One square centimeter "window" for the plate was cut out where the putative clone lined up with the film spot.

3. The areas corresponding to the signal was picked up by punching out the plaque using a 1ml pipette tip with cut end.

4. The phages from the agar plug were allowed to diffuse into 1ml of SM buffer containing a drop of chloroform for overnight at 4°C.

Plaque Purification: All the plaques showing positive signals from primary screening were purified and enriched by four rounds of screening and finally the positive plaques were stored in SM buffer with a drop of chloroform at 4°C.

2.2.8 Preparation of Plate Lysate

Plate lysate was made according to Sambrook et al., (1989). All the positive and purified plaques were titered first by spotting method as described earlier and depending on that titer 10^5 pfu were taken per plaque for plating on 82mm plate as described below.

1. 10^5 pfu of bacteriophage were taken and diluted with SM buffer upto 100μl and mixed with 300μl of freshly grown kw251 (A600=0.4) incubated at 37°C for 30 min.

2. After the adsorption of phages on host, it was mixed with 3ml of molten top agarose (maintained at 48°C) and plated on freshly prepared and dried LB-agar plate.

3. Plates were allowed to dry at RT and then incubated at 37°C till the complete lysis was seen.

4. 5ml of SM buffer was poured on the surface of each plate containing confluent lysis and kept at 4°C to allow the complete diffusion of recombinant phages in SM buffer for overnight.

5. Next day the plates were gently shaken at 100rpm at RT for 2 hr.

6. This freshly prepared liquid plate lysate was pooled in separate tubes. Each plate was washed with 1ml more SM buffer and mixed with 5ml plate lysate.
7. 0.1ml of Chloroform was added per tube, vortexed briefly and spun down at 4000g at 4°C for 10 min.
8. The supernatant was taken in a fresh tube and kept with a fresh drop of chloroform in each tube at 4°C as final plate lysate.
9. All the plate lysates were again titered by spotting method.

2.2.9 Isolation of DNA from Recombinant Phages

Phage DNA from the 16 positive plaques was isolated by the method described in Sambrook et al., (1989) with few modifications.

Preparation of Host Cells:
1. KW251 cells were streaked from glycerol stocks onto LB medium containing 15µg/ml tetracycllin, incubated at 37°C for overnight.
2. Single colony was inoculated in 25ml preculture of LB supplemented with 0.2% maltose and 10% MgSO4 and grown at 37°C at 200rpm for overnight.
3. For each plaque 100ml LB was taken in 500ml flask supplemented with 5mM CaCl2.
4. Each culture was inoculated with 1ml of preculture and grown at 37°C at 200rpm till A600 reached upto 0.75, thus 6X10^10 cells/100ml.

Infection at High Multiplicity:
1. Each culture was infected at a multiplicity of infection (moi) of 10 i.e.10^9 pfu of each clone from plate lysate and continued with shaking at 37°C at 200rpm until lysis occurred. It took 8-9 hr for complete lysis.
2. Lysis was completed by adding 1/10th the suspension volume of chloroform and left shaking at 37°C for another 30min. Lysed culture was stored at 4°C.

Purification of Bacteriophage:
1. To the cool lysed culture DNaseI (DN25 sigma) was added at a final concentration of 2µg/ml and RNaseI at a final conc. of 1µg/ml and incubated at RT for 2 hr.
2. 100ml of each culture was transferred to 250ml GSA bottle and 5.8gm NaCl(sigma) was added to achieve 1M final concentration; dissolved by swirling and left on ice-H2O bath for 4 hr (NB: The addition of NaCl promotes dissociation of bacteriophage particles from bacterial debris and is required for efficient precipitation of bacteriophage particles from polyethylene glycol).
3. Removed debris by centrifugation at 11000g for 10 min at 4°C.
4. Transferred supernatant in fresh bottle and solid PEG8000 was added to it at a final concentration of 10% (w/v), dissolved by swirling, left at room temperature for 30 min for complete dissolution of PEG, then left at 4°C for overnight.
5. Phage/PEG pellet was recovered by centrifugation at 11000g at 4°C for 10 min.
6. Bacteriophage pellet from each 100ml culture was resuspended in 3ml TM buffer (TM buffer-10mM Tris-HCl pH 7.6, 10mM MgCl₂, 20mM NaCl) and transferred in 50ml centrifuge tube; again the wall of the GSA bottle was rinsed with another 6ml of TM to minimize phage loss.

7. Polyethylene glycol and cell debris were extracted from the bacteriophage suspension by the addition of equal volume of chloroform.

8. Aqueous phase was separated by centrifugation at 3000g for 15 min at 4°C. The interphase was reextracted with another 3ml of TM and pooled in with the aqueous phase.

9. Supernatant containing the phage particles was treated with proteinaseK at a final concentration of 50μg/ml along with 20mM EDTA and 0.5% SDS. Mixed gently and incubated at 58°C water bath for 1 hr.

10. All the tubes were cooled at room temperature for 1 hr and extracted with equal volume of phenol:chloroform twice by centrifugation at 3000g for 5 min at RT.

11. The aqueous phase was then extracted with chloroform:IAA once under same condition.

12. DNA was precipitated from the supernatant by the addition of Na-acetate at a final concentration of 0.3M and 2 volume ethanol at RT for overnight.

13. DNA pellet was recovered by centrifugation at 12,000g at 4°C for 5 min, washed with 70% ethanol, and vacuum dried.

14. DNA pellet was dissolved in 300μl TE (pH 8.0).

15. Yield of DNA was quantitated by agarose gel electrophoresis with serial dilution of purified λDNA as standards.

2.2.10 Restriction Mapping of Lambda Clones

250ng of DNA from all 16 positive plaques was taken in 10μl reaction volume for each restriction digestion. Restriction digestions were set up using buffers provided by the restriction enzyme manufacturer (mostly NEB if not mentioned otherwise) at the recommended temperature.

**Determination of Insert Sizes**: Insert sizes of all positive clones were determined by the single digestion with BamHI and SfiI independently and the digests were transferred onto Duralon UV membrane and probed with labelled 1.2kb EcoRI fragment of the cDNA insert. Depending on BamHI digestion data clones were categorized into different groups. Finally, the clone λAG5 having the largest insert was taken for fine mapping with BglII, Clal, EcoRV, Ncol SphI with respect to BamHI.

2.2.11 Subcloning

Depending on the double digestion data of λAG5 clone different restriction digestions were carried out to subclone the total insert into different sizes of fragments.
Vectors used  --  BamHI cut dephosphorylated pBSIIKS+
                   BamHI and EcoRV cut pBSIIKS+
Inserts used   --  8 kb BamHI-EcoRV fragment of λAG5
                   --  3.6 kb BglII fragment of λAG5
                   --  2.09 Kb BglII-BamHI fragment of λAG5
                   --  1.4 kb BamHI fragment of λAG5

Preparation of BamHI Cut, Linearised, Dephosphorylated Plasmid Vector:

1. 1.1 μg of pBSIIks+ plasmid DNA was digested to completion with BamHI in a 15 μl reaction volume at 37°C.
2. The linearised plasmid (2.96 kb) was gel eluted by using Geneclean II kit as per manufacturer instruction and estimated by agarose gel.
3. 500 μg of the purified linearised plasmid was dephosphorylated in a 15 μl reaction volume using calf Intestinal phosphatase (promega) at 37°C, for 30 min using CIP buffer.
4. The reaction was stopped by adding EDTA to a final conc. of 5 mM and CIP was heat inactivated at 75°C for 10 min.
5. The linearised and dephosphorylated vector was again gel purified as described above.

Preparation of Double Digested Plasmid Vector with BamHI and EcoRV

1. 1 μg of pBSIIks+ plasmid DNA was initially digested to completion with BamHI in a 10 μl reaction volume at 37°C.
2. The linearized DNA was gel-purified and quantitated by agarose-gel.
3. The linearized DNA was then digested with the second enzyme EcoRV in a reaction volume of 10 μl at 37°C to completion.
4. The double digested linearized plasmid DNA was gel-purified.

Preparation of Inserts from λAG5 Clone:

(a) Preparation of 1.4kb BamHI Insert
1. 6 μg of λAG5 genomic DNA was digested to completion with BamHI in a 100 μl reaction volume at 37°C.
2. The 1.4 kb BamHI fragment was gel purified and quantitated by agarose gel.

(b) Preparation of 3.6kb BglII Insert and 2.09kb Bgl-II BamHI Insert
1. 2.25 μg of λAG5 genomic DNA was double digested with BamHI and BglII in 50 μl reaction volume at 37°C.
2. Both 3.6 kb and 2.09 kb bands were gel purified by using Geneclean II kit and quantitated by gel.
(c) Preparation of 8.0kb EcoRV-BamHI Insert
1. 2.25µg of λAG5 genomic DNA was double digested in a total volume of 50µl at 37°C.
2. The insert was gel eluted and quantitated by agarose gel.

Ligation
(a) 12.5µg BamHI cut linearized, and dephosphorylated vector per reaction was used for ligation of 1.4 kb BamHI fragment, 3.6 kb BglII fragment and 2.09 kb BglII-BamHI fragment. The inserts quantities were 10ng, 16ng and 16ng respectively.
(b) 20ng of double digested BamHI-EcoRV cut vector was used with 65ng of 8 kb BamHI-EcoRV insert for ligation.

All the ligations were performed in ligase buffer (NEB) containing 3wu of T4 DNA ligase in a total reaction volume of 20µl at 16°C for 12-16 hr.

2.2.12 Transformation of E. Coli

Preparation of DH5α Competent Cells: DH5α was tested for recA as described in Maniatis et al. (1982). DH5α competent cells were made following the method of Alexander, (1987), with some modifications.

Steps in the Procedure
1. DH5α cells were streaked on fresh LB-agar plate from glycerol stock.
2. Single colony was inoculated in a preculture of 10ml of 2X Luria broth (2% Bactotryptone, 1% Yeast extract, 0.1% NaCl) medium supplemented with 0.2% glucose.
3. Preculture was grown at 30°C at 200rpm for 16 hr.
4. Culture was inoculated with 1/100th volume of preculture in a conical flask (NB: Culture volume should be 1/10th of the capacity of the flask) in 2XL supplemented with 0.2% glucose.
5. Culture was also maintained at 30°C at 200rpm till A600 reached 0.45.
6. The bacterial culture was then chilled in a ice-H2O bath for 2 hr.
7. Cells were pelleted at 2500g for 15 min at 4°C.
8. Pellet was resuspended in freshly made acid-salt buffer (100mM CaCl2, 70mM MgCl2, 0.04M NaOAc, pH 5.5) at 1/2 the volume of starting culture volume. Acid salt buffer was filter sterilized.
9. Resuspended pellet in acid-salt buffer was incubated on ice H2O bath for 45 min.
10. Cells were pelleted by centrifugation at 2500g for 10 min at 4°C.
11. Competent cells were then resuspended in acid salt buffer in a 1/20th volume of the starting culture volume.
12. Competent cells were mixed with sterile glycerol to a final concentration of 15% (v/v).
13. Cells were aliquoted on ice and frozen at -80°C in eppendorf tubes and stored at the same temperature till further use.

This method yielded competent cells with the transformation efficiency of 10⁷-10⁸ cfu/µg DNA.
Transformation: For each transformation, 100 μl of competent cells was used.

1) Competent cells were thawed completely on ice.
2) 20 μl of each ligation mix was made up to 50 μl by addition of 30 μl TE (pH 8.0).
3) This 50 μl ligation mix then added to 100 μl of competent cells on ice and incubated for 45 min on ice with gentle tapping at regular intervals.
4) The cells were subjected to heat shock at 37°C in a water bath for 5 min and then stored on ice.
5) 150 μl of cells were then added to 3.8 ml of 2×L broth and cells were allowed to outgrow for 1.30 hr at 37°C at 200 rpm to allow the expression of the antibiotic resistance gene.

Cells not receiving any plasmid DNA and the uncut vector were used as controls. Transformation efficiency was calculated as the number of transformed colonies/μg DNA compared to the transformation efficiency of the uncut vector.

Plating of Transformation Mix: 40 μl of 20 mg/ml X-gal (Promega) and 4 μl of 200 mg/ml IPTG (BRL) was spread on 20 ml LB-agar plate containing 75 μg/ml ampicillin. An aliquot of 200 μl out of 4 ml transformation mix was plated in each plate. The plates were dried in laminar flow air and then incubated at 37°C incubator, agar side up till the colonies appeared (16 hr). The plates were then stored at 4°C until blue, white coloured colonies become distinct.

2.2.13 Screening and Analysis of Recombinants

24 recombinant colonies (white), from each transformation set were picked up and patched on a fresh LB-agar master plate containing ampicillin and simultaneously inoculated into 1.5 ml TB medium containing 50.0 μg/ml ampicillin. The cells in liquid culture were grown at 37°C in an incubator shaker at 200 rpm for 16-18 hr. The master plate was incubated at 37°C incubator till the patched colonies appeared.

2.2.14 Isolation of Plasmid DNA and Restriction Analysis

Plasmid DNAs from each putative clones were isolated by miniprep plasmid isolation and analysed by restriction digestions.

2.2.14.1 Small Scale Plasmid DNA Isolation

Small scale plasmid DNA isolation was done by modified boiling method of Sambrook et al., (1989).

1. 1.5 ml culture was taken into each microfuge tube. Cells were pelleted at 12,000 g for 20 sec at RT.
2. Medium was removed by aspiration completely leaving the bacterial pellet as dry as possible.
3. Bacterial pellet was resuspended thoroughly by vortexing 500μl of freshly made STET (0.1M NaCl, 10mM Tris-HCl, pH8.0, 1mM EDTA, pH 8.0 and 5% Triton X100).
4. 40μl of 20mg/ml freshly made lysozyme was then added to each tube.
5. Tubes were placed in a boiling H2O bath exactly for 1 min and allowed to cool at RT for 5-10 min.
6. Bacterial lysate was centrifuged at 12,000g for 7 min at RT.
7. The pellet of bacterial debris was removed using sterile toothpick.
8. Nucleic acid was precipitated by the addition of 1/10th volume of 3M Na-acetate, pH 5.2 and equal volume of isopropanol to the supernatant. Mixed by inversion and incubated at RT for 15-30min.
9. Nucleic acid pellet was recovered by centrifugation at 12,000g for 5 min at RT.
10. Supernatant was removed by gentle aspiration, pellet was washed with 70% ethanol and vacuum dried.
11. Pellet was dissolved in 25μl TE, pH 8.0.
12. DNA concentration was estimated spectrophotometrically at A260 and the purity was measured by A260/280 ratio.

2.2.14.2 Medium Scale Plasmid DNA Isolation
Midipreparation of plasmid DNA from 50ml TB culture was done by the protocol described in Sambrook et al., (1989).

1. Single colony from LB-agar plate containing appropriate antibiotic was inoculated in 50ml TB medium supplemented with the same antibiotic. The cells were grown overnight (16-18 hr) at 200 rpm at 37°C Shaker. In case of low copy number plasmid DNA isolation, Chloramphenicol mediated amplification was done as per the reference cited above.
2. Before starting the DNA isolation 1ml of bacterial culture was stored with glycerol at a final conc. of 15% (v/v) in -80°C. Cells were harvested at 4000g at 4°C for 15 min.
3. The cells were resuspended in freshly made 2.4ml of lysis buffer (25mM Tris-HCl, pH 8.0, 10mM EDTA, pH 8.0, 50mM glucose).
4. After thorough suspension lysozyme was added at a final concentration of 1mg/ml and incubated on ice H2O for 10 min.
5. 4.8ml of freshly prepared 0.2N NaOH, 1% SDS was added to this. The lysate was mixed by gently inversion and incubated on ice for 10 min.
6. 3ml of 3M NaOAC, pH 5.2 was added and mixed thoroughly by inversion. The tube was stored on ice for 20 min.
7. The lysate was centrifuged at 12,000g for 15 min at 4°C and the supernatant was transferred to a fresh tube.
8. Nucleic acid was precipitated by addition of 0.6 volume of isopropanol at RT.
9. After 15 min incubation at RT nucleic acid was recovered by centrifugation at RT at 12,000g.
10. The nucleic acid was washed with 70% alcohol and dried at vacuum.
11. The nucleic acid pellet was dissolved in 4ml of TE, pH 8.0.
12. DNase free RNase A was added at a final concentration of 20μg/ml and incubated at 37°C for 30 min.
13. The sample was extracted twice with an equal volume of phenol: Chloroform, mixed with gentle inversion, centrifuged at 12,000g for 15 min to separate the phases at RT.
14. The aqueous phase was reextracted with equal volume of chloroform:IAA at the same condition as above.
15. The aqueous phase was transferred to fresh tube and nucleic acid was precipitated with Na-acetate, pH5.2 at a final conc. of 0.3M and 2 volume of ethanol at -20°C for 30 min.
16. Nucleic acid was pelleted at 12,000g at 4°C for 10 min and washed with 70% alcohol and vacuum dried.
17. The dry pellet was dissolved in 400μl MQ H2O transferred into 1.5ml tube and the plasmid DNA was purified by NaCl and PEG at a final conc. of 0.8M and 6.5% respectively; mixed by gentle inversion and incubated in ice-H2O bath for one hour.
18. Plasmid DNA was pelleted at 12,000g at RT for 15 min; washed with 70% alcohol and dried under Speed vac.
19. The plasmid DNA was dissolved in 200μl TE, pH 8.0 and estimated spectrophotometrically. The routine yield was 200-400μg.

2.2.15 Generation of Unidirectional Nested Deletions with Exonuclease III

Unidirectional nested deletions of each of the subclone in opposite orientations was generated by the method of Henikoff, (1987).

Preparation of Supercoiled Plasmid DNA: To get closed circular DNA, nicked and linear DNA population from the midipreparation of DNA (section 2.2.14.2) was selectively removed by acid phenol extraction according to promega protocol.

Preparation of Acid Phenol: 500ml of redistilled phenol with 0.1% hydroxyquinolene was mixed with equal volume of 50mM Na-acetate (pH 4.0) and shaken at RT for 3-4 hr. After phases got separated, upper aqueous phase was removed by aspiration and rest of the phenol was reextracted with equal volume of 50mM Na-acetate (pH 4.0), stirred vigorously to emulsify. Upper aqueous phase was again removed and pH was checked using pH paper. This extraction was continued repeatedly till pH attained 4.0. Acid-equilibrated phenol was stored at 4°C.

Reagents:
- 2M Na-acetate, pH 4.0
- 3M Na-acetate, pH 5.2
- 1M Tris-HCl, pH 8.6
- 2M NaCl

Acid Phenol Extraction: DNA from midipreparation was used as the starting DNA for acid phenol extraction.
1. DNA was precipitated with 0.1 volume of 3M Na-acetate, pH 5.2 and 2 vol. of ethanol at -20°C for 30 min.

2. DNA pellet was recovered by centrifugation at 12,000g for 15 min at 4°C; washed with 70% ethanol and vacuum dried.

3. DNA pellet was dissolved in sterile MQ H2O. 2M Na-acetate, pH 4.0 was added to DNA solution at a final conc. of 50mM and 2M NaCl was added to give a final concentration of 75mM and mixed by finger tapping.

4. DNA solution was extracted twice with equal volume of acid phenol and centrifuged at 10,000g for 5 min.

5. To the upper aqueous phase 0.05 vol. of 1M Tris-HCl, pH 8.6 was added and extracted with equal volume of chloroform: IAA.

6. Aqueous phase was then precipitated with 0.1 vol. of 2M NaCl and 2 vol. of ethanol at -80°C for 30 min.

7. Supercoiled DNA pellet was recovered by centrifugation at 12,000g for 10 min at 4°C.

8. Pellet was washed with 70% alcohol, vacuum dried and finally dissolved in 100μl TE, pH 8.0. All the steps in acid-phenol purification was carried out in cold room and on ice. The DNA prepared by this method was used for deletion immediately.

Unidirectional Nested Deletions:

Solutions required:

- 10X ExoIII buffer -- 660mM Tris-HCl, pH 8.0, 6.6 mM MgCl2
- 7.4X S1 buffer -- 0.3M KOAc, pH 4.6, 2.5mM NaCl, 10mM ZnSO4, 50% glycerol.
- S1 stop mix -- 0.3M Tris-base, 0.05 M EDTA.
- Klenow buffer -- 40mM Tris-HCl, pH 8.0, 100mM MgCl2.
- dNTP mix -- 0.125mM each of dATP, dTTP, dGTP and dTTP.
- 10X ligase buffer (NEB).
- 25% PEG 8000

Deletion of pSBG 3.6F and pSBG3.6R:

1. 10-12μg of recombinant plasmid was cleaved with KpnI followed by EcoRI. A small part of the reaction mix was analysed on 0.7% agarose gel to check for completion of digestion.

2. The double digested plasmid DNA was extracted with equal volume of TE saturated phenol:chloroform followed by chloroform:IAA and then precipitated with 2 vol. of ethanol. Care was taken to minimize vortexing the sample to prevent any nicking of the DNA samples.

3. DNA pellet was rinsed in 70% alcohol, vacuum dried and resuspended in 30μl MQ H2O, DNA was quantitated spectrophotometrically.

4. For 3.6 kb insert, 18 timepoints were taken.

5. Microfuge tubes were labelled according to the timepoint and placed on ice water.
6. S1 reaction mix was prepared as follows:
   7.4X S1 buffer -- 18.5μl
   S1 Nuclease, 5u/μl -- 9.0μl
   MQ H2O -- 107.5μl
   135.0μl
   7.5μl of S1 reaction mix was dispensed into each of the labelled tubes on ice.

7. ExonucleaseIII reaction mix was prepared as follows:
   Double digested DNA - 18.0μl (4.5μg DNA)
   10X ExolIII buffer - 4.5μl
   MQ H2O - 21.0μl
   * ExolIII 100u/μl - 1.5μl (150u)
   45.0μl
   * added after removing no enzyme control.

8. 2.5μl of ExolIII reaction mix was added into S1 mix of the tube marked as no enzyme control.

9. Prewarmed the ExolIII reaction tube in heat block set at 37°C for 5 min.

10. 1.5μl (150u) of ExolIII was added and quickly mixed with pipette tip while continuing the incubation at 37°C.

11. 2.5μl aliquots were removed at every 30 sec with a fresh tip and mixed with 7.5μl of S1 reaction mix on ice.

12. The tubes were given a brief spin and the samples incubated at 25°C for 15 min for S1 nuclease to act. 1μl of S1 nuclease stop buffer was added and S1 nuclease was heat inactivated at 70°C for 10 min in presence of 10mM EDTA.

14. 3μl of each timepoint was analysed on 0.8% agarose gel to see the extent of deletion. Double digested starting plasmid and vector alone were used to compare the extent of deletions. The size of the deleted fragments per time point was estimated using λ-HindIII as standard.

15. The tubes were preincubated at 37°C for 5 min and then 1μl of klenow mix was added to each sample and incubated at 37°C for another 5 min.
   klenow buffer -- 20.0μl
   klenow (5u/μl), NEB -- 2.5μl (12.5u)
   22.5μl

16. To each tube 1μl of dNTP mix was added and tubes were incubated at 37°C for another 5 min. The tubes were then chilled on ice.

17. Ligase reaction mix was prepared as follows:
   10x Ligase buffer -- 80.0μl
   25% PEG -- 16.0 μl
   T4 DNA Ligase (6.6wu/μl) -- 3.0μl
   MQ H2O -- 557.0μl
   800.0μl
   40μl of the above mix was added per sample and incubated at 20°C for 8 hr.
18. 10μl of each ligation mix was used to transform DH5α competent cells.

For doing deletion of pSBG2.09F, pSBG2.09R, pSBG1.4F, pSBG1.4R; all the reactions were performed as was done in case of pSBG3.6 F and pSBG3.6R with exceptions mentioned below.

1. For deletion of pSBG1.4F and pSBG1.4R HindIII-KpnI double digested DNA was used as template, for pSBG2.0F and pSBG2.09R EcoRI-KpnI double digested DNA was used.
2. For pSBG1.4F and pSBG1.4R 6 time points were taken whereas as for pSBG 2.09F and pSBG 2.09R, 9 time points were taken.
3. Ratio of DNA quantity and ExoIII remained same for all as was in pSBG3.6F and pSBG3.6R.

Screening of Deletion Subclones: Screening of each deletion subclones were done by cracking method as described in Promega protocol.

Solutions required:
- 10mM EDTA, (pH 8.0)
- 2X Cracking buffer (0.2N NaOH, 0.5% SDS and 20% sucrose)
- 4M KCl
- 0.4% Bromophenol Blue.

1. Using sterile toothpick, individual colonies from transformation plate was picked up and smeared in 25μl of 10mM EDTA, (pH 8.0) and then patched simultaneously onto a LB-agar plate with ampicillin.
2. Cells were resuspended by vortexing.
3. 25μl of 2Xcracking buffer was added to each tube and vortexed. Tubes were incubated at 70°C for 5 min and then allowed to cool at RT.
4. 0.75μl of 4M KCl and 0.25μl of 0.4% bromophenol blue was added per tube; vortexed to mix.
5. 20μl of each sample was analysed in 0.8% agarose gel and desired deletion subclones were chosen from this assay.
6. To analyze the extent of sequence overlap form the deletion inserts, miniprep DNA isolation of desired subclones were done and digested with PvuII. The inserts that gave 300-400 bp deletions with a 50 bp overlap were chosen for sequence analysis.

2.2.16 Double Stranded DNA Sequencing by Dideoxy Chain Termination Method

The nested deletion subclones were sequenced using Cy5™ Auto Read™ sequencing kit (Pharmacia) by the dideoxy chain termination method of Sanger et al., (1977).

2.2.16.1 Preparation of Double Stranded DNA Template and Sequencing Reactions: Double stranded plasmid DNA was prepared by medium scale plasmid DNA isolation protocol described in section 2.2.14.2.
Sequencing Protocol:

Solutions needed:
- 2M NaOH
- 3M NaOAc, (pH 4.8)
- 70% ethanol
- 100% ethanol
- 5'cy5 reverse primers: 5'cy 5'-d [CAGGAAACAGCTATGAC]3' in aqueous solution, 2.1μM (2.1pmol/μl).

Annealing buffer: a buffered solution containing MgCl2
Enzyme dilution buffer: a buffered solution containing glycerol, BSA, DTT
Extension buffer: a buffered solution containing DTT
DMSO
T7 DNA polymerase: In buffered glycerol solution
A mix: ddATP in solution with dATP, dCTP, C7d GTP and dTTP
C mix: ddCTP in solution with dATP, dCTP, C7d GTP and dTTP
G mix: ddGTP in solution with dATP, dCTP, C7d GTP and dTTP
T mix: ddTTP in solution with dATP, dCTP, C7d GTP and dTTP
Stop solution: Deionized formamide/Blue dextran

Standard Annealing of Primer to Double Stranded Template

1. Using sterile MQ H2O, the concentration of the template DNA was adjusted so that 32μl contained 10μg DNA.
2. To this 32μl (15μg) template DNA, 8μl of 2N NaOH was added at a final conc. of 0.4N and was mixed gently, briefly centrifuged and incubated at RT for 10 min.
3. 7μl of 3M Na-acetate (pH 4.8) and 4μl of MQ H2O was added to each tube.
4. DNA was precipitated by the addition of 120μl of 100% ethanol, mixed gently and incubated at -80°C for 1-2 hr. Pellet was recovered at 12,000g at 4°C for 15 min.
5. Pellet was washed with 70% alcohol, and dried under vacuum.
6. Dry pellet was dissolved in 10μl MQ H2O.
7. To each tube containing 10μl denatured template DNA 2μl of CY5 reverse primer (4.2 pmol) was added along with 2μl of annealing buffer. The components were mixed by gentle finger tapping and centrifuged briefly.
8. The annealing reaction was preheated at 65°C for 5 min and immediately transferred to 37°C, incubated 10 min.
9. Tubes were removed from 37°C and incubated at RT (25°C) for another 10 min, centrifuged briefly.
10. 1μl of extension buffer and 3μl of DMSO was added per tube and the sequencing reaction was done immediately.
Sequencing Reactions:

Essential preliminaries

* T7 DNA polymerase dilutions: Using the cold dilution buffer supplied in the kit T7 DNA polymerase (8u/μl) was diluted as 4u/μl. Mixed gently by pipetting and stored on ice until use.

* Aliquoting of A-mix, C-mix, G-mix and T-mix: 4 eppendorf tubes were labelled as A,C,G, and T respectively. 2.5μl of each mix was pipetted into corresponding labelled tubes and stored on ice. (The aliquoting was done during 10 min incubation of annealing reaction at 37°C).

After the annealing reactions was cooled to room temperature, the tubes were centrifuged briefly to ensure that all the components are at the bottom of the tube.

Reactions:

1. Four sequencing reaction mixes just dispenced was warmed by placing the tubes at 37°C heatblock for atleast 1 min before going to the second step.

2. 2μl of diluted enzyme was added to the annealing reaction and mixed thoroughly with pipette tip, immediately 4.5μl of this mixture was added into each of the prewarmed sequencing mixes at 37°C using each time a fresh pipette tip for each addition.

3. Reactions were incubated at 37°C for 5 min.

4. 5μl of stop solution was added to each reaction and mixed by gentle agitation, briefly centrifuged.

The reactions were stored at 4°C until the sequencing gel was ready.

2.2.16.2 Casting Sequencing Gel

Solutions required:  

<table>
<thead>
<tr>
<th>Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethanol 96% not denatured</td>
<td>Acrylamide 5.7% (w/v)</td>
</tr>
<tr>
<td>Acetic acid 10%</td>
<td>N,N'-Methylenebisacrylamide 0.3% (w/v)</td>
</tr>
<tr>
<td>Bind-Silane, Plus one</td>
<td>Urea 7M</td>
</tr>
<tr>
<td>Bind-silane, working solution</td>
<td>Tris-borate (pH8.3) 100mM</td>
</tr>
<tr>
<td>- 1ml ethanol, 3μl Bindsilane</td>
<td></td>
</tr>
<tr>
<td>mix and 250μl 10% acetic acid</td>
<td></td>
</tr>
<tr>
<td>(freshly made)</td>
<td></td>
</tr>
<tr>
<td>Detergent - Extran (neutral pH)</td>
<td></td>
</tr>
<tr>
<td>Readymix gel, A.L.FTM Grade-</td>
<td></td>
</tr>
<tr>
<td>supplied by Pharmacia</td>
<td></td>
</tr>
<tr>
<td>Component</td>
<td>Concentration</td>
</tr>
<tr>
<td>--------------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Na$_2$EDTA</td>
<td>1mM</td>
</tr>
<tr>
<td>N,N,N',N'-Tetramethylethlenediamine</td>
<td>3mM</td>
</tr>
<tr>
<td>10XTBE</td>
<td>242.28g Tris Base, 102.64g Boric Acid, 7.44g EDTA/2l.</td>
</tr>
<tr>
<td>10%APS</td>
<td>Prepared fresh by dissolving 0.1g/ml MQ H$_2$O.</td>
</tr>
<tr>
<td>TEMED</td>
<td>Pharmacia</td>
</tr>
</tbody>
</table>

Routinely 4.8% denaturing sequencing gels were run. The gel composition was:

- **Ready mix gel, A.L.F.TM grade**: 60.0ml (4.8%)
- **10XTBE**: 6.0ml (0.8X)
- **MQ H$_2$O**: 9.0ml
- **75.0ml**

300μl of 10% APS
and 30μl of TEMED.

DNA sequencing was done using ALF expressTM sequencing gel apparatus from Pharmacia. Spacers and comb used were 0.5mm thick.

### Assembly of the Cassette:

1. Both thermal plate and the glass plate were washed thoroughly with dish brush soaked in detergent solution. The plates as well as the upper buffer tank (attached its the thermal plate) were rinsed thoroughly with warm tap H$_2$O followed by MQ H$_2$O. Combs and spacers were also cleaned in similar fashion.

2. All the components were air-dried and then wiped out with 70% alcohol to remove any dust particle.

3. 100μl of Bind silane working solutions was applied with paper towel at the top of the thermoplate and 5cm down from the top of the glass plate.

4. The upper 2cm of thermoplate and upper 5cm of glass plate was then polished with paper towel.

5. Treated areas were rinsed with MiliQ H$_2$O and wiped dry.

6. Both the plates were polished with paper towel soaked in ethanol by overlapping vertical strokes from bottom and ended with the bind silane treated areas.

7. Cassette was assembled following the ALF express manual and clamped tightly.

The gel was cast on a labelling plate as soon as APS and TEMED were added and gel was allowed to polymerize 3-3.5 hr before loading the samples.

### 2.2.16.3 Running Conditions

The gel was run with 0.5XTBE at 1500V, 60mA 25w, at 55°C. The sampling interval was 2 sec. and running time was 600 min. The samples were denatured by heating at 85°C for 3 min, spun briefly and chilled on ice before loading. The surface of the gel and each well was flushed with a syringe to remove any deposited urea. 6-7μl each sample was loaded per lane within 30 min from the start. Before starting the final run laser beam was aligned carefully for
dust particles and for reflections. The cassette was leveled adjusting each wheel equally and the water circulation was also connected.

2.2.16.4 Sequence Storage and Reading

The sequence was stored by AM version 3.0 software in each 2 sec interval in the directory C:/AMDATA/. The sequence was then read through OS/2 operating system using ALF express computer and print outs were taken with peak of each nucleotide.

The overlaps between the successive subclones were established by the use of Genepro programme in IBM. Finally the sequence was compiled using PC-Gene sequence analysis Package (Intelligenetics).

2.2.16.5 DNA Sequence Analysis and Homology Search

The sequence of the various subclones was compiled after comparing the sequence of the opposite strand. The DNA sequences were compared to the sequences available in the EMBL (Ham and Cameron, 1986) and Genebank (Bilofskye et al., 1986) databases. Conserved sequences for eukaryotic promoter elements and terminator was found out by using PC Gene and Gene-runner programmes. Homology of the deduced aminoacid sequences was searched for all the amino acid sequences in the Swiss-PROT (Bairoch and Boeckmann, 1991) database using fast A program (Pearson and Lipman, 1988).

2.2.17 PCR Amplification of Genomic DNA and Fine Mapping

As most of the dicot storage protein genes have been reported to have one or more than one intron in the coding region, it was of interest to find out the number and position of intron/s in AmAl gene, if any, by PCR amplification of genomic DNA.

2.2.17.1 Designing of Primer

3 forward primers at different positions of cDNA sequence and one reverse primer were made for use in PCR (Saiki et al., 1988).

<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Position in cDNA</th>
<th>Length</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>F51</td>
<td>47-64</td>
<td>22 mer</td>
<td>70</td>
</tr>
<tr>
<td>F-353</td>
<td>353-370</td>
<td>18 mer</td>
<td>50</td>
</tr>
<tr>
<td>F415</td>
<td>415-432</td>
<td>18 mer</td>
<td>50</td>
</tr>
<tr>
<td>F-658</td>
<td>658-675</td>
<td>18 mer</td>
<td>50</td>
</tr>
<tr>
<td>O-3B</td>
<td>90-110</td>
<td>21 mer</td>
<td>56</td>
</tr>
<tr>
<td>R-1044</td>
<td>1044-1062</td>
<td>23 mer</td>
<td>68</td>
</tr>
</tbody>
</table>

All the positions have been marked in respect to pAmAl.3 sequence.
Sequences
F-51 5' CACCATGGCCGATTACC 3' (The oligo corresponding to ATG codon with 4 nucleotides 5' overhang was synthesized. ATG is underlined).
F-353 5' GTAGAAGAAGGTAACATG 3'
F-415 5' AAATTATACCGTGGTGG 3'
F-658 5' TGTCATCTGATGGTGC 3'
O-3B 5' TGATATCTTAAGTACTTCTGG 3'
R-1044 5' CAAGGAAGAACCTCTTTTTCC 3'

Synthesis and Purification of Oligos: All oligos were synthesized by using oligo synthesizer (model 391) from Applied Biosystems by trityl off method as per instruction manual.

Post Synthesis: Coupling efficiency was determined by checking the trityl fractions. The eluent fractions 1.85ml (trityl) each was mixed with 8.15ml of p-toluenesulphonic acid in acetonitrile (CDH), and 1ml of each fraction was taken for O.D. estimation at A530.
(NB: 9.5g of p-toluenesulphonic acid was dissolved in 500ml acetonitrile, filtered through 1mm whatman and used). In all the cases coupling efficiency was 98%.

Manual Deprotection and Cleavage: Oligonucleotides were simultaneously decyanoethylated and cleaved from the support using concentrated ammonium hydroxide, followed by the removal of base protecting groups by the addition of fresh concentrated ammonia and incubation at 55°C.

Reagents, Solvents and Apparatus: 1ml disposable syringes with luer fittings, disposable needles with female leuers, rubber stopper, male to male luer connectors, concentrated ammonium hydroxide, DNA collection vials with a teflon lined caps.

1. After the oligo synthesis was complete the column was removed from the instrument and one end of the column was attached to the syringes luer. Male to male luer was connected at the other end of the column and the syringe needle was attached to the male to male luer.
2. With the syringe plunger inserted into the syringe barrel to the 0.5ml mark, the needle was inserted into fresh room temperature equilibrated ammonium hydroxide. Enough ammonium hydroxide was filled in the column but the volume in the syringe was minimized.
3. The needle was inserted into the rubber stopper and kept standing for 15-30 min. This treatment begins cleavage and cyanoethyl deprotection.
4. Expelled the ammonia into a small DNA collection vial.
5. Ammonia treatment was repeated three more times for 15-30 min each. This ensures complete cleavage and cyanoethyl deprotection.
6. To remove the exocyclic ammonia base protecting groups, first the DNA volume was made 3ml with fresh concentrated ammonium hydroxide. Then the vial containing DNA was kept at 55°C for 17 hr to complete the cleavage.

7. 2ml of each sample was stored at 4°C in ammonia only, rest 1ml was processed further.

8. This 1ml sample was speedvac to evaporate ammonia, washed with MQ H2O and dried again by speedvac.

9. Finally, the pellet was dissolved in 200μl of MQ H2O and then precipitated with 1/10th vol. of 3M Na-acetate (pH 5.2) and 2 vol. of ethanol at -80°C.

10. Pellet was spun down at 12,000g for 5 min, washed with 70% ethanol and dried.

11. Finally the DNA was dissolved in 200μl TE (pH 8.0).

Size and Purity Determination of Oligos: Oligos were quantitated by spectrophotometer by taking O.D. at A260 and A280. In all the cases approximately 200μg DNA was there in processed sample. Size and the purity was checked by running oligos in a 20% denaturing PAGE (8.4g urea, 2ml 10XTBE, 10ml 40% acrylamide solution volume upto 20ml with 25μl 25% APS and 10μl of TEMED) along with known sized oligos as markers. Gel was run with 1XTBE buffer at 500 volt constant. In each case 2μg of oligo was mixed with stop solution (2.5X : 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and xylene cyanol) at a concentration of 1X and loaded onto gel after heating at 95°C for 1 min and quick chilling. All oligos were stored at -20°C for long term storage.

2.2.17.2 PCR Amplification of Genomic DNA Using F-51 and R-1044

PCR reactions were performed in a 25μl reaction volume according to the specifications of the suppliers (Perkin Elmer). 350ng of genomic DNA was used as template with 10 pmole of each primer. Four sets were done, using no formamide, 1.25% formamide, 2.5% formamide and 5% formamide. A 25μl reaction without template was also performed simultaneously as a control. After initial denaturation for 2min at 95°C, 30 cycles were performed by denaturation at 95°C for 1min, annealing at 60°C for 1min and extension at 72°C for 2min . A final extension was done at 72°C for 10 min to complete the synthesis. PCR was performed in PTC-100-60 Thermal cycler of MJ Research, Inc, USA. 10μl of each sample was analysed on a 0.8% agarose gel along with λHindIII and pUC-Hinfl as DNA standards.

Using F-353 and R-1044 and F-615 and R1044: PCR reactions were performed exactly as mentioned for F-51 and R-1044. Only the annealing was done at 50°C instead of 60°C. The PCR product of F-51 and R-1044 was confirmed by southern hybridization using 1.02 kb EcoRI fragment of pAmAl.3 as probe.
2.2.17.3 Cloning of the PCR Product in T-Vector

Insert preparation: Using F51 and R1044 primers, amaranth genomic DNA was amplified in large scale (5x25μl reactions). The samples were pooled and then the PCR product was run on a 0.7% agarose gel. Band corresponding to the expected size of the insert was gel-purified and DNA was estimated again on agarose gel (30ng/μl in a total volume of 25μl TE, pH 8.0).


1. pBSIIks+ DNA (5μg) was digested with EcoRV in 50μl reaction volume for overnight (16 hr) at 37°C.
2. Digested DNA was precipitated with 0.5 vol. of 7.5M NH₄-acetate and 2 vol. of ethanol, mixed by gentle inversion and left at -80°C for 2 hr.
   The tube was centrifuged at 12,000g at 4°C for 15 min to recover the DNA pellet. DNA pellet was washed with 70% ethanol, dried under vacuum and dissolved in 20μl MQ H₂O.

Polymerase Reaction to Add T at 3' End: One extra T was added to the EcoRV cut pBSIIks+ linerized DNA by the use of Tao DNA polymerase.

Digested DNA          --  20μl
10XPCR buffer         --  10μl
MgCl₂ (25mM)          --  6μl (1.5mM)
BSA (1μg/ml)          --  20μl (2.0μg)
dTTP                   --  20μl (2mM)
Tao polymerase        --  1μl (5u)
MQ H₂O                --  41μl
                          --  100μl

100μl reaction was overlayed with 50μl of mineral oil and extension was done PCR machine at 70°C for 2 hr. The whole reaction mix was ran on 0.7% gel, the band corresponding to the amplified product was cut out and DNA was eluted in TE (pH 8.0) in a total volume of 50μl. DNA was quantitated by gel electrophoresis and estimated to be 100ng/μl.

Ligation: Ligation was set up in a 10μl volume with 100ng vector and 150ng of insert using 2 unit of T4 DNA ligase. The ligation reaction was kept at 16°C for 5 hr. 1μl of the ligation mix was used to transform DH5a competent cells as described in section 2.2.12. Transformants were selected on LB-agar supplemented with ampicillin and IPTG and X-Gal. 24 white colonies were picked up for miniprep DNA analysis as described in section 2.2.14.1.

Restriction Digestion of the Clones: HindIII and PstI double digestion was carried out for 15 putative transformants. BamHI digestion was also done to know the orientation of the
clones. Midipreparation of two clones (one in sense orientation and another in opposite orientation) were done as described in section 2.2.14.2.

2.2.17.4 Restriction Mapping of pSB4 and pSB5
The insert was restriction analysed by kpnI, SalI, HindIII, MspI, PvuII, XbaI, Asel, BamHI, EcoRI, HincII, PstI, Apal, Clal, Ndel and SmaI. In each case 200ng of DNA was digested in a volume of 10µl.

2.2.17.5 Sequencing of pSB4
To do sequencing, unidirectional nested deletion was done using supercoiled plasmid DNA of pSB4 after digesting with SalI and XbaI as described in section 2.2.15. 17 time points each of 30 sec interval were taken for deletion. Screening of deletion subclones were performed as mentioned in section 2.2.15. Insert sizes were found out by PvuII digestions. DNA sequencing reactions were done by dideoxy chain termination method using circumvent TM Thermal Cycle dideoxy DNA sequencing kit with vent (exo-) DNA polymerase as per NEB instruction manual using 35S dATP (BARC, India, specific activity 600ci/mmol).

2.2.17.6 Manual Sequencing Gel
Casting Sequencing Gel

Solutions required:

<table>
<thead>
<tr>
<th>Solution A</th>
<th>Solution B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silane A174(Pharmacia)</td>
<td>Acetic acid</td>
</tr>
<tr>
<td>Ethanol</td>
<td>MQ H2O</td>
</tr>
<tr>
<td>25.0µl</td>
<td>75.0µl</td>
</tr>
<tr>
<td>5.0ml</td>
<td>750.0µl</td>
</tr>
</tbody>
</table>

Total solution A was mixed with 150µl solution B and used. 30% acrylamide stock (19:1) was prepared and deionized as recommended (Maniatis et al., 1982). Routinely 5% sequencing gel was ran. The gel composition was:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>10XTBE</td>
<td>15ml</td>
</tr>
<tr>
<td>30% acrylamide</td>
<td>25ml</td>
</tr>
<tr>
<td>Urea</td>
<td>63g</td>
</tr>
<tr>
<td>H2O</td>
<td>60ml</td>
</tr>
<tr>
<td></td>
<td>150ml</td>
</tr>
</tbody>
</table>

25ml of gel solution was used for sealing the bottom of the gel apparatus before pouring the gel with 100µl of 25% APS and 100µl of TEMED. Rest 125ml gel solution was polymerised using 100µl of 25% APS and 50µl of TEMED for casting the gel. Seqi-Gen Nucleic acid sequencing cell (Bio-Rad) was used for running the gels. Plates used were 38X50cm dimension. Spacers used were 0.4mm thick, 77 well sharkstooth comb 0.4mm thick were used to form the wells.
One of the plates bonded to the buffer chamber was treated with repel silane. The outer longer plate was treated with bind silane. The assembled plates were sealed at the sides by clamps and at the bottom with a strip of whatman 3MM paper impregnated with catalysed acrylamide. The gel was cast after degasing the gel solution.

Electrophoresis: The gels were preran at 2000v, 100w for 2-3 hr until the gel temperature stabilized at 50°C. The samples were denatured by boiling at 95°C for 3 min, spun briefly and mixed. The surface of the gel was flushed with a syringe to remove the undissolved urea. 2.5µl of each sample was applied to the top of the gel surface with sequencing gel loading tip. Each sample was given two loadings.

2.2.17.7 Reading the Sequence

The sequence was read from the bottom of the gel in the second loading and then into the first loading of the same reaction. The sequence was then compiled into one. The overlaps between the successive clones was established by using Gene Pro programme. The sequence was compiled using PC Gene sequence analysis package (Intelligenetics).

2.2.17.8 DNA Sequence Analysis and Homology Search

The sequence of the various subclones was compiled after comparing the sequence of the opposite strands. Differences were sorted out by going back to the autoradiogram. The DNA sequence was compared to the sequences available in EMBL (Ham and Cameron, 1986) and Gene bank (Bilofskye et al., 1986) databases and homology of the deduced aminoacid sequences in the Swiss PROT (Bairoch and Boeckmann, 1991) database using fast A program (Pearson and Lipman, 1988).

2.2.18 Gel Mobility Shift Assay

2.2.18.1 Preparation of Nuclear Extracts

Nuclear extracts from stem, young seeds and old seeds were prepared according to the protocol of Itoh et al., (1993) and Mikami et al., (1994) with few modifications. All procedures were performed at 4°C in cold room using pre-cooled buffers and equipments. Either freshly grown materials or frozen tissues at -80°C were taken for preparing nuclei.

1. Tissues were ground with a pre-cooled mortar and pestle under liquid nitrogen to fine powder.
2. Powdered tissue was suspended in modified Honda buffer at 10 times w/v ratio (Modified Honda buffer : 2.5% ficoll-400, 5% dextran T-40, 25mM Tris-HCl, pH 8.5, 5mM MgCl₂, 2.5% TritonX 100, 0.44M sucrose, 10mM β-ME, and 2mM spermine).
3. The powdered tissue was homogenized using polytron for 1.5 min, three times each for 30 sec with 1 min cooling interval.
4. Slurry was filtered through double layers of cheesecloth and miracloth.
5. Nuclei were pelleted at 2500g for 5 min at 4°C.
6. Pellet was washed with modified Honda buffer without spermine.
7. Nuclei were lysed in an equal vol. (w/v) freshly made nuclei extraction buffer (20mM Hepes-KOH, 420mM NaCl, 1mM DTT, 0.2mM EDTA, 25% glycerol, 1mM PMSF, 5μg/ml antipain and 5μg/ml leupeptin) by incubating on ice for 45 min.
8. Nuclear extract was centrifuged at 18,000g for 30 min at 4°C.
9. Supernatant was dialysed against 50 fold vol. of cold dialysis buffer (20mM Hepes-KOH, 50mM KCl, 20% glycerol, 0.5mM DTT, 0.2mM EDTA and 1mM PMSF) for 4 hr at 4°C with three changes of buffer.
10. The clear nuclear extract recovered after centrifugation of dialysed supernatant was aliquoted into small fractions and stored at -80°C.
11. Protein conc. was determined using Bio-Rad protein assay reagent.

2.2.18.2 Preparation of 32P-labelled DNA Probe

A deletion subclone of pSBG3.6F (5-0-9F) was used as target for gel mobility shift assay.

Isolation of Inserts: (i) 75μg of DNA was digested in 450μl vol. with HindIII-AflII in NEB recommended buffer. (ii) 2μg of 0.387 kb HindIII-AflII fragment was again digested with AseI to release 0.255 kb AseI-AflII fragment in 40μl reaction vol. Both the fragments were resolved in 0.8% agarose gel and purified using Geneclean II kit. The yield of each insert was determined by comparing the fluorescence with that of known amount of DNA standard.

Target DNA Labelling: 100ng of both 0.387 kb and 0.255 kb fragments were end-labelled in 50μl reaction vol. using klenow polymerase. A typical reaction contained 100ng of DNA, 200μM dNTPs excluding the one used as radiolabelled nucleotide, 10-20μCi α32PdATP or dCTP or both, and 2.5u of klenow polymerase in 50μl vol. containing klenow buffer. The reaction was carried out at 25°C for 30 min and klenow was heat inactivated at 75°C for 10 min. The samples were extracted with equal vol. of phenol:chloroform and chloroform:IAA. DNA was purified over sephadex G-50 spun column and then precipitated with 0.5 vol. of 7.5M NH4-acetate and 2.5 vol. of ethanol at -80°C for 1 hr. DNA pellet was washed in 70% alcohol, vacuum dried and resuspended at a conc. of 2-5ng/μl in TE, pH 8.0.

2.2.18.3 DNA-Protein Binding Reactions

DNA protein binding reactions were carried out in a 20μl vol. containing 2-5ng of probe DNA in binding buffer (20mM Hepes-KOH pH 7.5, 50mM KCl, 0.2mM EDTA, 1mM DTT, and 5% glycerol). 20μg of nuclear extracts were used per reaction. 0.5-2μg poly dl-dC (Pharmacia) and salmon sperm DNA were used per reaction. The reactions were preincubated at 25°C for 10 min before addition of labelled probe. After the preincubation, probe was added to the reaction and incubated at same condition for another 20 min.
2.2.18.4 Electrophoresis of DNA-Protein Complexes

The protein-DNA complexes were resolved on a 5% non-denaturing PAGE (19:1 acrylamide:bis-acrylamide) in 0.5X TBE. The samples were ran on a 1.5mm thick gel in Hoefer apparatus. Gel was pre-ran at 100V for 1 hr. After completion of pre-run samples were loaded and ran at 150V for 2-3 hr at RT.

2.2.18.5 Detection of DNA-Protein Complexes by Autoradiography

The gel was transferred onto a whatman 3mm sheet, covered by saran wrap on the top and was vacuum dried in a gel drier for 3 hr. The dried gel was exposed to Kodak X-Omat AR film with intensifying screen at -80°C.

2.3 Results and Discussions

2.3.1 Genomic Southern and Copy Number

Genomic DNA digested with different restriction enzymes revealed on the autoradiogram single band where insert does not have any site for the enzyme or 2 bands in the case of a single sites in the insert and 3 bands wherever insert has 2 sites for the enzyme(Fig. 4A and 4B). Thus, AmA1 gene is a single copy gene present in Amaranthus hypochondriacus genome. It is a rare occurrence where a seed storage protein is encoded by a single gene. The mechanism of spatial and temporal regulation of this AmA1 storage protein gene would be relatively easy and interesting to find out as AmA1 is encoded by a single copy gene.

2.3.2 Genome Size

To find out the approximate genome size of Amaranthus hypochondriacus, a quantitative method was used. Sphl-BamHI digestion of pAmA1.3 releases a fragment of 0.621 kb. Different amounts (2pg, 4pg, 6pg, 8pg, 10pg, 12pg, and 18pg) of 0.621 kb were made from a digestion reaction by serial dilution. Genome size was determined from southern blot resulted from known quantity of genomic DNA digested with Sphl-BamHI that would give a 0.621 kb fragment against the same fragment of serially diluted plasmid DNA (Fig.5). An absolute value for the genome size was determined from densitometric scanning of autoradiogram by comparing the relative band intensity. Since AmA1 is a single copy gene, the genome size was calculated as 1.035 x10^6 kb.

2.3.3 Choice of λGEM12 as a Cloning Vector

Genomic library of A. hypochondriacus was constructed in the phage λGEM12. A 1.183 kb EcoRI fragment representing the cDNA insert was cloned previously by screening a λgt11 expression library using affinity purified AmA1 antibodies (Raina and Datta, 1992). Since it was of interest to clone the entire gene, a replacement vector was chosen. The chromosome of phage lambda is a linear DNA molecule, 48.6 kb long of which 40% is unessential for propagation of the phage. This permits manipulation of the lambda genome without affecting
Fig. 4: Genomic southern hybridization. A Amaranth genomic DNA digested with different enzymes (HaeIII: Ha, HindIII: H, Pvull: P, Sphl: Sp, Xbal: X, Sacl: S, Clal: C, Ncol: N, BamHI:B and EcoRI: E). U stands for undigested DNA. B. 2μg of digested DNA for each sample was transferred to Duralon UV membrane and probed with 1.12 kb EcoRI fragment of cDNA clone.
Fig. 5: Genome size estimation. 5μg of SphI-BamHI digested genomic DNA (G) was electrophoresed into 0.7% agarose gel along with different dilutions (2-18 pg) of SphI-BamHI digested pAmA1.3; blotted onto Genescreen Plus membrane and probed with 0.9 kb BamHI, fragment of pSB2.
its packaging and propagation in the host. The strategy of replacement vector involves digestion of the phage DNA with appropriate restriction enzymes which would generate a left arm, a right arm which would contain all the information essential for production of infective phage particles and a central stuffer fragment which is indispensable. The fragments to be cloned are digested with compatible restriction enzymes and fractionated to provide fragments in the range of 8-20 kb (replacement with exogenous DNA that results in genomes of the range 40-50 kb are packaged with high efficiency). The partially filled-in XhoI half site arms bypasses the size fractionation step by partial fill-in of insert genomic DNA fragments. Ligation of such DNA molecules with the arms of the vector would generate a variety of molecules flanked by the left and right arms. When introduced into a bacterial cell such a molecule would enters the lytic cycle and produces a clone of viral particles. To generate a complete library, the genomic DNA was cleaved by partial digest of Sau3A1 to provide fragments with random cleavage sites such that overlapping fragments are represented in the library (Fig.6). In λGEM12, the central stuffer region is flanked by polycloning sites having sites of SacI, NolI, BamHI, EcoRI, XhoI and XbaI (Fig.7). Generation of partials of genomic DNA with Sau3A1 and partial fill-in permits cloning into the compatible partially filled-in XhoI half site arms (Fig.8). The phage DNA, thus, produced could be packaged in vitro using packaging extracts which are prepared following the induction of E.coli lysogenic for mutant derivatives of lambda. The packaging extracts contain all the structural proteins required for the assembly of lambda DNA into infective phage particles. The phages adsorb the receptor on the surface of E.coli through the tip of the tail fibres. These receptors are encoded by lamB gene and their production is stimulated by the uptake of maltose from the medium. The adsorption is temperature dependent and facilitated by the presence of Mg$^{2+}$ ions. To prevent the adsorption of phages to dead cells, cells from log phase (A$_{600}$ = 0.45-0.55) are used. Preadsorption synchronises the infection and reduces the heterogeneity in plaque size.

### 2.3.4 Screening the Genomic Library

The genomic library in λGEM12 represented 2-3 genome equivalents of *A. hypochondriacus* (taking into consideration average insert size in lambda clones are 15 kb and *A. hypochondriacus* genome size is 1.035x10^6 kb). To obtain a full length clone 1.78x10^5 recombinants (2-3genome equivalents) of the primary library were plated on LB-agar plates which were one-two days old. To obtain even distribution of plaques, the plates were poured on a leveled surface. The plates were incubated upside down to prevent condensation collecting on the agar surface. Plaques appeared after incubation for 6-8 hr at 37°C as clear areas against a background of unlysed cells. The plates were immediately stored at 4°C to prevent the top layer of agar from peeling off while lifting the plaques on nylon membranes. 18 positive signals were obtained of which 2 were very faint and weak and hence, 16 positive plaques were punched out of the agar using a cut 1ml autopipette tip. The phages were allowed to diffuse into SM buffer. The plaques were repurified by further rounds of dilution and plating. For secondry screening 5000 plaques were plated on 82mm plates, for tertiary screening 1000 plaques and for final quaterenary screening 100 plaques were plated to give well isolated independent plaques. Plaques were purified until all the phages that were
Fig. 6: Sau3Al partial digestion of genomic DNA. A. Optimization of Sau3AI partial digestion lane1-1 μg of amaranth genomic DNA was subjected to partial digestion with 2u (lane 2), 1u (lane 3), 0.5u (lane4), 0.1u (lane5) of the enzyme, and resolved onto 0.5% agarose gel. Lane 1 represents 1μg of undigested genomic DNA. B. Large scale preparation of Sau3AI partial digestion. 1μg of genomic DNA was subjected to partial digestion with 3u (lane 1), 4u (lane 2); electrophoresed onto 0.4% agarose gel. HindIII cut λDNA was used as molecular weight DNA marker.
Fig. 7: Simplified map of λGEM12 vector. The 20 kb left arm and 9 kb right arm are separated by the non-essential stuffer fragment (14 kb). The multiple cloning site was inserted between the λarms and stuffer region. The cos-sites in the left and right arms are indicated in the figure.
Fig. 8: XhoI half-site arms cloning strategy of λGEM12: Generation of partial fragments of genomic DNA with Sau3A1 and partial fill-in permits cloning into the compatible partially filled-in XhoI half site arm.
plated gave positive signals. During screening the intensity of the signal varied among the plaques but the intensity was consistent throughout the screening procedure. After quaternary screening 16 positive plaques were obtained. The results of the final round of screening are shown in Fig.9. The 16 positive plaques were named as λ.AG1 to λ.AG16.

2.3.5 Relatedness of the Genomic Clones

Phage DNA was isolated from 16 positive plaques. The insert sizes were determined by digestion of DNA from all of the plaques with EcoRI which flanks the cloning site in the left and right arms. The average insert size was found to be 15-16 kb (Fig.10A). To determine the relatedness of the clones, the DNA of the clones were digested with BamHI, since a single site of BamHI was present in the 1.183kb EcoRI fragment used as a probe. The southern data showed the presence of a common 10 kb or 6.5 kb fragment (Fig.10B). 10 plaques belonged to category 1 and one plaque each belonged to category 2,3,4 and 5. The insert sizes of each of these plaques (as they occur in the genome) is given in Table-7. One of the plaque λ.AG5 from category-2, which showed 2 flanking fragments of BamHI site was used for detailed restriction analysis. Single and double digestions of BamHI, BgIII, ClaI, EcoRV, NcoI, SphI were used for restriction mapping after doing southern hybridization (Fig.11).

2.3.6 Subcloning

The BamHI-BamHI (10 kb), BglII-BglII (3.6 kb), EcoRV-BamHI (2.09 kb), BamHI-BamHI (1.4 kb) inserts of λ.AG5 plaque were subcloned into pBSIIks+ phagemid (short et al., 1988 and Alting-Mees and Short, 1989). All the inserts were subcloned in two opposite orientations to facilitate the sequencing of the opposite strands. The advantages of using pBSIIks+ to subclone was due to the presence of convenient restriction sites in the polylinker to generate deletions and the presence of lacZ gene which facilitates blue-white color selection for recombinants.

2.3.7 Generation of Unidirectional Nested Deletions and Sequencing

Several strategies are available for the division of large DNA fragments into smaller regions for sequencing. Most methods rely on the availability of convenient restriction sites or subcloning of random fragments. Both the methods often result in generating gaps which are difficult to fill. The other widely used method involves the use of oligonucleotide primers and relies on the availability of sequence information of at least a part of the subclone. The use of ExoIII depends on the presence of convenient restriction sites between the insert and the binding site for the sequencing primer, the enzyme that cleaves closer to the insert and leaves a 5' overhang or blunt end; and one that cleaves further from the insert and leaves a 4-base 3' overhang (Henikoff, 1987). As ExoIII does not attack a 4-base 3' overhang, the deletions proceed unidirectionally. Since, the rate of deletion is sensitive to temperature, it is possible to adjust the rate of deletion by varying the temperature. To obtain 300 bp fragments with a 50 bp overlap between subsequent fragments, 18 time points were chosen for a 3.6 kb fragment (Fig.12), 9 time points were chosen for a 2.09 kb and 6 timepoints for a 1.4 kb fragment.
Fig. 9: Final screening of the λGEM12 genomic library with radioactive 1.2 kb DNA probe. 100 phages of 16 positive plaques were screened per clone. The numbers indicate the plaque numbers λAG1 to λAG16.
Fig. 10: Southern blot analysis of relatedness of the lambda clones. 250 ng of DNA from each clone was digested with EcoRI (A) and BamHI (B), separated on 0.8% agarose gel. Numericals represent the digests of respective genomic clones λAG1-λAG16. DNAs were transferred onto Duralon UV membrane and probed with radiolabelled 1.2 kb EcoRI cDNA insert.
Table 7: Map Of The BamHI Inserts Of The Genomic Clones Aligned As They Occur In The Amaranth Genome

<table>
<thead>
<tr>
<th>Clone Numbers</th>
<th>Sizes of BamHI Inserts (in Kb)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ. AG1</td>
<td>10</td>
</tr>
<tr>
<td>λ. AG2</td>
<td>10</td>
</tr>
<tr>
<td>λ. AG3</td>
<td>10</td>
</tr>
<tr>
<td>λ. AG4</td>
<td>10</td>
</tr>
<tr>
<td>λ. AG5</td>
<td>10, 1.5</td>
</tr>
<tr>
<td>λ. AG6</td>
<td>10</td>
</tr>
<tr>
<td>λ. AG7</td>
<td>6.9, 2.2</td>
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<tr>
<td>λ. AG8</td>
<td>10</td>
</tr>
<tr>
<td>λ. AG9</td>
<td>10</td>
</tr>
<tr>
<td>λ. AG10</td>
<td>10</td>
</tr>
<tr>
<td>λ. AG11</td>
<td>10, 0.21</td>
</tr>
<tr>
<td>λ. AG12</td>
<td>10</td>
</tr>
<tr>
<td>λ. AG13</td>
<td>9.5</td>
</tr>
<tr>
<td>λ. AG14</td>
<td>9.5</td>
</tr>
<tr>
<td>λ. AG15</td>
<td>10.0</td>
</tr>
</tbody>
</table>

* Sizes of the right and left arm carrying a part of the insert are not given.

Clones λ. AG1, 2, 3, 4, 6, 8, 9, 10, 12, 15 are classified as category 1; clone λ. AG5 is classified as category 2; λ. AG7 is classified as category 3; λ. AG11 is classified as category 4 and λ. AG13 and λ. AG14 are classified as category 5.
Fig. 11: Detailed restriction analysis of λAG5 by southern hybridization. 250 ng of λAG5 was digested in each reaction. Single and double digestions of λAG5 were carried out with respect of BamHI. Gel was transferred onto Duralon UV-membrane and hybridized with 1.2 kb EcoRI fragment of cDNA clone of AmA1. EcoRV-ERV, BamHI-EcoRV-B+ERV, BglII-Bg, BamHI-BglII-B+Bg, BamHI-SphI-B+S, Clal-C, BamHI-Clal-B+C, Ncol-N, and BamHI-Ncol-B+N.
Fig. 12: Unidirectional nested deletion of 3.6 kb BglII subclones of pBSIIKS+ (pSBG 3.6). 4.5 µg of supercoiled plasmid was used as a template for ExoIII deletion. Deletion was allowed to proceed unidirectionally at 37°C and samples were withdrawn every 30 sec. The extent of deletion was monitored on a 1% agarose gel using starting plasmid and pBSIIKS+ as molecular weight markers. The size was estimated from the HindIII cut DNA as molecular size standard (lane 1-single cut pSBG 3.6; lane 2: 2-18, 18 different deletion time points; lane 19-single cut pBSIIKS+.
Deletions were performed at 37°C which gave a rate of 450 bp/min. Caution was exercised to use plasmid preparations in which the major population of molecules were supercoiled to prevent smearing of the deletions as ExoIII can actively digest nicked molecules to near completion. Screening of the deletions gave more than one kind of size of insert for any given time point. It was, therefore, imperative to check for the presence of the primer binding sites in the deleted sample by using a restriction enzyme site close to the primer site. Samples that had at least 50 bp overlap were chosen for sequencing. The deletion subclones used for sequencing are shown in (Fig.13).

For sequencing, double stranded plasmid DNA was used as a template. Both the strands were sequenced completely. To obtain the overlaps between BglII, BamHI subclones, EcoRV-BamHI subclone was sequenced. The sequence of the total gene of AmAl is presented in Fig.14 and 15.

2.3.8 PCR Cloning

Oligos were designed by using the programme PCR Plan of PC-Gene Package. Tm was calculated by the nearest-neighbour method. The 98% coupling efficiency showed that all the base addition was perfect. The 20% denaturing PAGE data revealed that all the oligos were pure and showed single band on gel compared to the standard oligos.

PCR Amplification and Effect of Formamide: Low concentration of non-ionic detergent formamide >10% have no adverse effect on the incorporation activity of Taq polymerase, though this concentration of formamide can change Tm, and thus increases the specificity of amplification by changing the effective stringency of the annealing step (Sarkar et al., 1990). This denaturant appears to improve the specific annealing of primer to template and so reduces mispriming. It has been proposed by Cheng et al., (1994) that these reagents enhance specificity of PCR by lowering melting and strand separation temperature which in turn facilitates denaturation of the template and increases the specificity of primer annealing. In fact, some reactions may amplify only in the presence of such additives (Pomp and Medrano, 1991). In case of amaranth genomic DNA different primers gave different results. PCR amplification data showed the presence of intron in the coding region of AmAl (Fig.16) and also it was confirmed that position of intron could be anywhere between F-51 and F-353 primer in the sequence as the amplified product of F-353 and F-658 gave expected 0.709 kb and 0.404 kb bands respectively though F-51 gave 2.5 kb band instead of expected 1.015 kb band (Fig.17A and 17B).

2.3.9 Sequence Analysis

The total sequence of both the strands was compiled using PCgene sequence analysis package (Intelligenetics). The total nucleotide length of pSB4 is 2551 bp. Analysis of sequence revealed a 912 bp open reading frame which encodes for a 35 kDa AmAl protein. The coding region of AmAl is interrupted by a single large intron of 1.55 kb. Intron starts with 2971 position and ends at nucleotide position 4501. The Intron showed the common consensus
Fig. 13: Sequencing strategy of *AmAT* genomic clone and its flanking regions
Fig. 14: Autoradiogram of the sequencing gel showing a part of the nucleotide sequence of 2.09 kb subclone, pSBG 2.09. Sequencing reactions of the deletion subclones were performed using Sequenase version 2.0 kit and (35s) dATP. Each sample was given two loadings in succession and resolved on a 6% denaturing gel in TBE buffer.
Fig. 15: Nucleotide sequence of the full length genomic clone of AmA1 with its flanking sequences. The sequence of both the strands was compiled from the deletion subclones using PC-Gene (Intelligenetics) sequence analysis software. The putative TATA box, other putative upstream sequence motifs, branch point sequence of the intron and the 5' and 3' splice junctions are highlighted on the sequence. Intron sequence is in italics. The start codon ATG and the stop codon TAA are in bold.
HaeIII
TTATTGATAATGGACCGGCCCAAACACTTTTTTAATAAAAGCGGTCTCAAGTAAGAATT
AATAACTTATCCTGGCAGGTTTTGTGAAATAATATTTCGCCAGAGTCTTTAA

GTGAATCCTCAGATATATATTTTTGTAAGGAAATTTCCACATGAGCATCCAGTTTATACAC
CATTTAGCGTTATATAAAAAATCTCCCTTTAAAGTCACTCGTAGTAGCTAAAATATCTGT

TATCTAAATTGCCGATGACATATTACCTGGCCGGGGTTGAAAAAAATATTTTCGCCAGAGTTCATTCTTAAA
GTGAATCTGCAATATATTTGGTAAAGGGAAATTTCACATGGTAGCATCCAGTTTATACAC
CACTTAGACGTTATATAAAAACATTCCCTTTAAAGTGTACCATCGTAGGTCAAATATGTG

TTGTAAGTTTTTTCCACATTGTCGATCCAGTTTTTTGTAAAATTCCACTCACTTTTTGACGTTT
AACATTCAAAAAAGTGTAAACATCGTAGGGTGCAACAATTTAAAGTGGTTAAAACGTCAA

TATTACCAATTTTTATTGTTAATTCACGGCTTTAAATCTTACGCTCAAATAGTGTGTTTAAAT
ATAAGGTGTTAAAAATAAACATGGAAGGGAAATTGCAGTAGTTCAAAAACAT

AAATTATGGAATTTAAAAATTATAATTAAATAGTGGTTATTTTACTGCAAATTTAAACTATTAA
TTAAAATACCTTTTTAATTATTTAACAATTTTTTAAAATAGCAGTTTTTTAAATTGAAAT

TTCCCATATTATGTTATACATCGTGCAAAACACAGATTATCTTTTGCATAAAGAAGTTACCTG
AAGGGGTATAATATTGTTTAATTCACCGTTTAATTCCTTAACGTCCATAAGTGTTTAAAT

ATAAGTGTTAAAATAACAAATTTAAGTGCTAATATTAAAATAAGTGTTAAAATAACAAATTTAAGTGCT

TTTTGATCGAATTTTCGAAAGAGTTATTCCATAAGGATAGTAGTGACATTCGTTTTTTCCA
AAAACTAGCTTAAAAGCTTTCTCAATAAGGTATTCCTATCATCACTGTAAGCAACCAAAAAGT

AAAAATGCGTATAATACATTTTTCGGTTTACATATTCCG
AAAAAGATCGTTATGTCAGGTAAAAATGACGCCAAATATC

TTTTAGCAAACTACATCCCCCATTTTTTACTGCCAGTTATGCTAAATCTTTCTCAATAGGGTATTCCTAT
ATTGCACGGTCTGTCTTCTTTTCTGACCCCTTTTTACTGCCGTTTTTATTATA

ATTGCACGGTCTGTCTTCTTTTCTGACCCCTTTTTACTGCCGTTTTTATTATA

AAAATAGGGGATAGTACCTTTTTTCGACGACAAAGGTTATTTAATTCCAATAGGGAATATCC
TTTATCGCTATTCATGAAACAGTGGGCTAAAATCTTTCTCAATAGGGAATATCC

ATTGCACGGTCTGTCTTCTTTTCTGACCCCTTTTTACTGCCGTTTTTATTATA

AAAATAGGGGATAGTACCTTTTTTCGACGACAAAGGTTATTTAATTCCAATAGGGAATATCC
TTTATCGCTATTCATGAAACAGTGGGCTAAAATCTTTCTCAATAGGGAATATCC

CIA
TTTTATCGATTTACGTATCGAAAAATAGGCGAAAAAGTACTTGTTTTCGACCGGATTAGAT
TAAAATAGCTAAAATGCGATCTTTTTATCCTGGCTTTTTTCTAGCAATACTACAGTCCATTTTTACTCGGTTTTATA

NsiI
AGTTTTCTTTAAAATTTTCAATAAAAATATTAAACATTTTTCTTTCGCTTAATGCTAACAG
TAAATAGCTAAAATGCGATCTTTTTATCCTGGCTTTTTTCTAGCAATACTACAGTCCATTTTTACTCGGTTTTATA

GTGAATCACAACATTAAAATGCGTAAATTTTAACAGAAATAAACTGAAAACACAGCTTAGCTTTTACCGATTAAATC
CATTGTTTTGTTTTTTACCCTTAATTTTGGCAATTTTTATTGCTTTAATTGGGACC

ATGCTACATCGGGAAAAAATTCTACAAACATGATGATTAAACAGGAAAATACAGAAGACTTT
TACGATGTGACCCCTTTTTGAAATGTTTTTACTACATTTAATTCGCTTTGAA
ACTTTTGATCTAGCAACCATTGGATTTGGATATTAAATATAAGGGAAATCCACATGGTA
TGAAAAACATGATCGTTGGATAACTAATCGTATTAAATTTTACCCTTTAAGTTGATCCAT

GCATCCAGTTTGTAGAATAACAGTGTGACACTTTTTTTAAAGAAATTTCCACATGGTAG
CGTAGGTCAAAAACATCTTTTACTGTCACACCTGTTGAAAATTTTTTAAAGTTGATCACAT

CATTCAATTCGATTTTTTTCGGAATGGTTGTTAATTCTCCACTTTAAATTTTATCTATTTTG
GTAAGTCAACCATCCTTTAAAAGGCAAATCAGAATATTAAAGTATTTAAATAGTAAAAC

TAAGTTTTTTCCACATGGTAGCATTCCAGTTTTTTTGTAAAATTTACCCTTTAACTCTAAAT
ATCAAAAAAGGGTGTACCTACGTTTGGTCAAAAATATAATGTTAAATGGGAAATGAAATA

TCACCATTTATGTTTTAATTCTCATTATTTAATTCATTTAAACGCTCACAATTTTAACTCTAA
AGTGGTAAAATTACAAATTTAACTGTAATTACAGGAATCTGTGTTGAAAATGAAATAAGG

TTTATTGAAAAATTATAAGAAAATATCATACTGCTTAACTGATCTTACATCGCTCTAT
AAATAACTTTTTAATATTCTTTTTATAGTTAGAGCCAGCTTTTTTCTTTAATACCCACGAT

TTTTGCTATTACGTTAACCAGGGAAAATGATTTTCTGGGTCGAGTTTTACCTATCATAGAAGTTT
AAAAGCTATGCTATTTTTCTTTTTTCTAAACCGCAGTCAAAAATGGTAAAATTGAGAATTAA

TCGAAAAACGGACGTTGCAATTACTCTTTATGGGATACCTTTTCTGAAAATCGGTCGAAAA
AGCTTTTTGCTTTGCAACGTATATGGAATATCTACATGAAAGAGGTTTTAAAGCCAGTTTTG

AGTACTTTATTCGCTTTATTTTTCCGATAGATGTGTTTCAAGAAAATGGGACCACTAAATGAT
TCATGAAATAACGGGATAAAAGGCCATCTCTACACCAAGTTTTTTACCTTTGTTGATTTAATA

HincII
TCAAAGGGGATTTTAGGATATTATGTTGACAAAAATTATATTGGAAACTATATAGCTATG
AGTTTATTCATCATAATCCTTACTTAATACAAATCTTTTAAATAAACACTTGATTATCGATAC

GTGATGCTTTGTCATTTTCTTACTGTTTTTGTCAACAATCATAAGTTTCTTGATCACATTTCC
CACTACGAAACAAGTTAAGGCTAATAAAACATGGTTGATTTTCAAGAAGACTAGTGTTAAAGG

AAAAATGAAATTTTATGGGCAAAAAATATGTAATATAAAAATTTGGCAACAGTCTATTTTA
TTTTTTTACTTTTTTTTTCACGTTTTTTTTATACATTTATTTTTAAACGTGTCGATAATTAAAT

AAAATTTTCACTTTTTTTTTTTTTTTTTAAAAGGCAAATACACATTAATATCTTTTATTTTGGGAT
TTTTAAAAGTGATTTTTTTTTTTTATTTTGGTATTATATGAAATAAATACCTTA

ATATTTTGTTGAAAATTTTAAATG1CAACCATTATAAAATTTGGTATAATACATATAGTT
TTAAAAACATTTTTAAATTTAACAGTGGTGATTTTTAAACATTTTATTGATATATCAA
Fig.16: Intron position in AmA1 along with the map of genomic clone (pSB4). PCR-amplification of AmA1 genomic DNA was done using the primers F-51 and R-1044. Total size of amplified product was 2.55 kb. Restriction mapping of pSB4 revealed a single large intron (1.5 kb) within 0.9 kb coding region of AmA1.
Fig. 17: PCR amplification of genomic DNA fragment of AmA1. PCR reaction was carried out using 350ng of genomic DNA of amaranth and resolved on 0.8% agarose gel. A. Lane 1 shows a single 2.55 kb amplified product; lane 2 represents negative control. B. Different reactions, without formamide (lane 2), with 1.25% formamide (lane3), 2.5% formamide (lane4) and 5% formamide (lane 5) were carried out. Lane 1 represents negative control.
sequence of 5' splice site GT and 3' splice site AG. Putative branch point sequence: CTTAT is found at position 60 nt upstream from the 3' splice site as predicted by Simpson et al., (1996).

Upstream of the translation start site consensus sequences like TATA are also present (Fig.15). The ORF begins with an ATG codon at nucleotide position 2757 and ends in TAA codon at position 5204. The coding region is flanked by 2.756 kb upstream sequence at the 5' end of ATG and 1.476 kb 3' untranslated region after TAA stop codon. Eukaryotic promoter element search was done using PCGENE programme. Different cis-acting and trans acting factor binding sequences are presented in the Table-8.

2.3.10 Gel Mobility Shift Assays

To investigate the presence of any cis-acting elements in 5' upstream of AmA1 which could bind any trans-acting factors to give seed specific expression, gel mobility shift assays (GMSA) were done. 0.387 kb HindIII-AflII and 0.255 kb AflII-AseI fragments were chosen due to presence of similar sequence homology of -300 element of monocot storage protein. Stem extract was used as a negative control in GMSA. The results indicate that both the fragments bind some trans-acting proteins from young and old seed extracts whereas in stem extracts no band shift was observed (Fig.18A and 18B). For both the DNA fragments 0.5μg of poly dI-dC or salmon sperm DNA showed higher shift. Further gel shift assays, using competitor DNA fragments and DNasel foot-printing assay would be required to pin-point the specific cis-sequences responsible for seed specific expression of AmA1.
Table 8: Putative Nucleotide Motifs Present In AmA1 Promoter Based On Computer Analysis

<table>
<thead>
<tr>
<th>Nucleotide motifs</th>
<th>Sites pattern</th>
<th>In AmA1</th>
<th>Putative Position</th>
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</thead>
<tbody>
<tr>
<td>TATA Box</td>
<td>TATA</td>
<td>TATA</td>
<td>-82 to -85</td>
</tr>
<tr>
<td>NF-E1</td>
<td>WGATAMS</td>
<td>AGATAAAG</td>
<td>-216 to -222</td>
</tr>
<tr>
<td>CREB</td>
<td>TGACGTCA</td>
<td>TGACGTCA</td>
<td>-1403 to -1410</td>
</tr>
<tr>
<td>SP1 Binding site</td>
<td>GGGCGG</td>
<td>GGGCGG</td>
<td>-2626 to -2631</td>
</tr>
<tr>
<td>Enhancer Seq for seed protein genes</td>
<td>(A/T/C)AACACA(A/C)(A/T/C)</td>
<td>CAACACAAG</td>
<td>-2699 to -2707</td>
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<tr>
<td>CACA Element</td>
<td>CCACCAAA</td>
<td>CCACCAAA</td>
<td>-542 to -549</td>
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<tr>
<td>-300 Element</td>
<td>TG(T/A/C)AAA(G/A) (G/T)</td>
<td>GTAAAAAT</td>
<td>-293 to -300</td>
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</table>
Fig. 18: Gel mobility shift assay for seed specific binding. Probe DNA used in (A) was 0.387 kb HindIII-AflII fragment of deletion subclone 5-0-9F of pSBG 3.6F and in (B) was 0.225 kb AseI-AflII fragment resulting from digestion of 0.387 kb fragment with Ase1. Protein-DNA binding reactions were done in a 20µl volume containing 2ng probe and 20 µg protein extract in each case. Numerals 0.5, 1, and 2 represents 0.5µg, 1µg and 2 µg of non-specific DNAs used per reaction. S - stem extract; YS-young seed extract; OS-old seed extract, F indicates free probe.