Plant Growth Conditions

Routine growth of Arabidopsis thaliana plants was at 21°C under a 16-hour photoperiod (long day) under fluorescent lights (7000 lux at 20 cm).

For growing the seeds in the pots: The synthetic soil mix used for growing plants in pots was prepared by mixing Soilrite (3:1 Peat: Perlite): Perlite: Vermiculite in a 1:1:1 proportion and soaked with 1X nutrient solution (Somerville and Ogren, 1982) or 1X MS before sowing seeds. For routine purposes 20-30 seeds were sown per pot (10 cm diameter). The pots were covered with saran wrap and shifted to a cold room (4°C) for 2-3 days before shifting them to the growth chamber.

For growing the seed on plates: The seeds were surface sterilized, first with ethanol for 2 min followed by treating them with 0.025% mercuric chloride for 3 to 5 min. Mercuric chloride was removed by washing the seeds thoroughly at least thrice with sterile water. The seeds were resuspended in luke warm 0.5% top agar (to avoid clumping) and plated evenly on MS agar (0.7%) media supplemented with 2% sucrose. For selection of Kanamycin resistant (KanR) plants Kanamycin was included in the MS media to a final concentration of 50 µg/ml. Before shifting the plates to light the seeds were stratified for 3 to 4 days at 4°C.

Plant growth conditions for the flowering time experiments

For the LD photoperiod conditions the plants were given 16 hrs of light and 8 hrs of dark. For the Short Day experiments the plants were grown under a cycle comprising of 8 hrs of light and 16 hrs of dark.

GA treatment: The plants were grown under SD conditions throughout the life cycle. 10 days after germination one set of plants was sprayed with 100 µM GA (Sigma) + 0.02 % Tween 20 (Sigma). GA was sprayed twice a week till the plants started to bolt. For control experiments one set of SD grown plants were sprayed with 0.02 % Tween 20 or were left untreated.
Plant Stocks used in this study

WT1A: Columbia (Col) background. These plants have a *glabra1* (*gl1*) mutation as a marker and hence lack trichomes. WT1A plants were used for LD, SD, GA treatment, anti-sense transgenic experiments.

Col: Wild type Columbia plants (with trichomes) were used for making Promoter–GUS reporter transgenics.

tfl-13 (Stock# CS6237) tfl-13 is an EMS induced mutant in Col background (with trichomes) received from ABRC. It is a strong allele of *tfl-13*, which shows early flowering, reduced plant height, determinate inflorescence and increased number secondary inflorescence. The phenotype is sensitive to photoperiod.

FRI-Sf2 (Stock# CS 6209) FRI-Sf2 is dominant, naturally occurring late flowering mutant allele in Col-2/Sf-2 background received from ABRC. The mutant plants flower significantly late when grown under LD conditions (75-90 days after germination).

SALK lines (Col-0, CS6000) ecotype was used for generating the T-DNA insertion mutants) T-DNA insertions in each of the AML gene were selected from the SALK T-DNA express database. These lines were received from Arabidopsis Biological Resource Center (ABRC), Ohio State University.

<table>
<thead>
<tr>
<th>S.No.</th>
<th>SALK LINES</th>
<th>AML gene disrupted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>SALK_015088</td>
<td>AML1</td>
</tr>
<tr>
<td>2.</td>
<td>SALK_061644</td>
<td>AML2</td>
</tr>
<tr>
<td>3.</td>
<td>SALK_029713</td>
<td>AML3</td>
</tr>
<tr>
<td>4.</td>
<td>SALK_006041</td>
<td>AML4</td>
</tr>
<tr>
<td>5.</td>
<td>SALK_019467</td>
<td>AML5</td>
</tr>
</tbody>
</table>

Table 6.1 List of SALK lines with insertions in individual AML genes
Materials and Methods

Bacterial Strains used in this study

E. coli (DH5α): F', ø80dlacZDM15, D(lacZYA-argF)U169, deoR, recA1, endA1, hsdR17(rlk<sup>−</sup>, mk<sup>−</sup>), phoA, supE44, l, thi-1, gyrA96, relA1

E. coli (HB101): supE44, hsdS20(r<sub>6</sub> - m<sub>6</sub> - ), recA13, ara-14, proA2, lacY1, gaiK2, rpsL20, xyl-5, mtl-1, leuB6, thi-1

Agrobacterium tumifaciens (AGL1): AGL1 carries the hypervirulent, attenuated tumor-inducing plasmid pTiBo542 from which T-region DNA sequences have been precisely deleted, allowing optimal DNA transformation of many dicotyledonous plants. Agrobacterium AGL1 also carries an insertion mutation in its recA general recombination gene, which stabilizes the recombinant plasmids (Lazo et al., 1991)

DNA Isolation

Plasmid preparation from E. coli

Routine plasmid preparation for checking the clones at different steps or cloning was done from a 3 ml overnight culture using alkali lysis protocol (Sambrook et al., 1989). Large scale plasmids used for the RNA-in situ probe preparation were made using the Qiagen50 tips, midi preps (according to manufacturer's protocol). Plasmids used for sequencing purposes were also prepared Qiagen tip20 mini prep column.

Plasmid preparation from Agrobacterium and BACs

For routine plasmid preprations 10 ml or 25 ml cultures were grown at 200 rpm at 28°C for 48 hrs. Plasmid was isolated by alkaline lysis protocol generally used for E. coli (Sambrook et al., 1989). The plasmid prepared by this procedure had a lot of polysaccharide contamination, which inhibited restriction enzyme digestion. To overcome this problem a CTAB purification step was included at the end of the alkaline lysis plasmid isolation procedure. After resuspending the DNA in TE, equal volume of 2 X CTAB was added, vortexed, and centrifuged at 13 K for 10 min. The top aqueous layer was transferred to a fresh eppendorf and an equal volume of chloroform: isoamyl alcohol (24:1) was added. The samples were centrifuged at 13K for 10 min. to separate the two phases and the aqueous phase was transferred to a fresh tube. For
precipitating the DNA 1/10 volume of 3 M sodium acetate and 2.5 vol. of ethanol were added, incubated at room temperature for 5 min followed by centrifugation at 13 K for 10 min. The pellet was washed with 70% ethanol; air dried and resuspended in 2 mM Tris (pH8).

**Genomic DNA isolation for Southern (Dellaporta Method)**

1-5 grams of fresh tissue was frozen in liquid nitrogen and ground into fine powder in a clean mortar and pestle. The powder was transferred into a 30 ml centrifugation tube (without allowing it to thaw) and 3 ml of freshly prepared extraction buffer (100 mM Tris (pH 8), 50 mM EDTA, 500 mM NaCl, 1.4 % SDS, and 10 mM mercaptoethanol) was added and mixed well. After adding equal volume of 2 X CTAB the samples were incubated at 65° C for 5 min. The cell debris was removed by centrifugation at 8000 rpm in an SS34 rotor for 10 min. The clear supernatant was collected into a fresh tube, an equal volume of Chloroform: Isoamyl alcohol (24:1) was added and mixed by gentle vortexing. Two phases were separated by centrifugation at 8000 rpm for 10 min and the upper aqueous phase was transferred to a fresh tube. To precipitate the DNA 2/3 volumes of isopropanol was added, mixed slowly by inverting the tubes a few times. The samples were incubated at room temperature for 30 min. before pelleting the DNA at 10,000 rpm. The pellet was air dried, resuspended in 400 µl of TE and transferred to a 1.5 ml eppendorf. Once again phenol: chloroform, followed by chloroform extraction was performed to ensure the removal of protein contamination. The DNA was precipitated by adding one tenth volume of 3M sodium acetate (pH 5.5) and 2.5 volumes of ethanol followed by centrifugation at 13000 rpm (in BIOFUGE tabletop refrigerated centrifuge) for 15 min. The pellet was washed twice with 70% ethanol and air dried before resuspending in 50 µl of TE.

**Isolation of DNA using Phytopure (Amersham)**

For PCR genotyping genomic DNA was isolated from small tissue samples using Phytopure (Amersham) according to the manufacturers protocol.

**Rapid plant Genomic DNA isolation by Liu’s Method**
Liu’s protocol (Liu et al., 1995) with slight modifications was used for rapid DNA isolation. A small piece of leaf was collected in a 1.5 ml eppendorf tube, and snap frozen in liquid nitrogen. The tissue was macerated using disposable pestles and 150μl of freshly prepared extraction buffer (50 mM Tris (pH 7.5); 300 mM NaCl; 20 mM EDTA; 0.5 % SDS; 2 % sarcosyl; 5 M urea; and 5 % phenol) was added. To remove the cell debris the samples were centrifuged at 13 K for 5 min. at room temperature and the upper clear phase was collected into a fresh tube. 2.5 vol. of ethanol were added and the tubes were incubated on ice for 10 min before pelleting the DNA at 13 K for 10 min. The pellet was washed twice with 70% ethanol; air dried for 5 min and resuspended in 30 μl of 2 mM Tris (pH.8). Two micro litre of this was used as a template in PCR. The DNA prepared by this protocol is not stable beyond a few of days (1 week).

Plant Genotyping
Southern analysis

For Southern hybridisations genomic DNA was isolated using the Dellaporta method, digested with restriction enzymes (restriction reactions were supplemented with 1mM Spermidine to facilitate complete restriction) and separated on 0.8% agarose gel. Further processing of the gel (depurination and denaturation) and transfer of DNA to Hybond N* Nylon membrane (by capillary transfer) was performed according to Sambrook et al., 1989. Hybridisations were performed in 7% SDS, 0.5 M Sodium Phosphate and 1 mM EDTA (Kevil et al., 1997) at 65°C for at least 16 hrs. Post Hybridisation, the blots were washed with 1 X SSC, 0.1 % SDS; 0.5 X SSC, 0.1 % SDS and finally in 0.1 X SSC, 0.1 % SDS at 65 °C for 30 minutes each.

The signal was detected by autoradiography or on Fuji BAS-1800 Phosphor imager. The L-Process and Image Gauge programs (Fuji) were used for quantitations and subtracting the background.

PCR based Genotyping
For genotyping SALK lines DNA was isolated using Liu’s DNA isolation protocol or using a shorter version of the Dellaporta protocol. Table 6.2 shows a list of primers used for genotyping the SALK lines.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer name</th>
<th>Primer sequence</th>
<th>T_m for the primers (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-DNA</td>
<td>LB1</td>
<td>aac cag cgt gga ccg ctt gct g</td>
<td>72.6</td>
</tr>
<tr>
<td>T-DNA</td>
<td>LB2</td>
<td>cag ggc cag ggc gtg aag g</td>
<td>69.6</td>
</tr>
<tr>
<td>AML1</td>
<td>K22F1</td>
<td>ggg agg aca gga gga caa cat tga</td>
<td>65.8</td>
</tr>
<tr>
<td></td>
<td>K22R1</td>
<td>cac ccc ccg att tac aga act cta gg</td>
<td>67.6</td>
</tr>
<tr>
<td>AML2</td>
<td>AML2F1</td>
<td>gct gaa aaa tta aca tgc aag caa gaa ac</td>
<td>66.5</td>
</tr>
<tr>
<td></td>
<td>AML2R1</td>
<td>ttt agt tgg tat atc cac tat ccc atg c</td>
<td>63.3</td>
</tr>
<tr>
<td>AML3</td>
<td>F7F1</td>
<td>ggt aag cgt tgt ctc gtt glt t</td>
<td>63.6</td>
</tr>
<tr>
<td></td>
<td>F7R1</td>
<td>caa tga gct tga caa gaa cat gct g</td>
<td>61</td>
</tr>
<tr>
<td>AML4</td>
<td>F15F1</td>
<td>cct gaa tca tgt cga tgc gcc ttt g</td>
<td>63.5</td>
</tr>
<tr>
<td></td>
<td>F15R1</td>
<td>cgc cga caa gaa gat gag aaa acc</td>
<td>61.9</td>
</tr>
<tr>
<td>AML5</td>
<td>T28F1</td>
<td>gat ggc aag aag gc agc aat gt</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>T28R1</td>
<td>aac cgt gtc cac tat ctt acc cat gt</td>
<td>65.7</td>
</tr>
</tbody>
</table>

Table 6.2
List of primers used for genotyping the SALK insertion lines for presence of the T-DNA and for identifying lines homozygous for the insertion in each of the AML gene

RNA Isolation and Expression Studies

Isolation of total RNA

RNA was isolated from plant tissue using Trizol (Gibco) as per the manufacturers instructions. The tissues (rosette leaves, inflorescence, and roots) were collected in a 1.5 ml eppendorf or a 15 ml falcon tube according to the amount of tissue to be collected. The tissue samples were snap frozen in liquid nitrogen for storage at -70°C until RNA isolation. Using disposable micropestles the frozen tissue was grounded to fine powder, the tissue was not allowed to thaw at any point of time until trizol was
Materials and Methods

Required amount of Trizol (1 ml per 100 mg tissue) was added, followed by vortexing the tubes for 1 min. To separate the cell debris samples were centrifuged for 10 min at 12 K, 4°C (Biofuge.pico, Heraeus Instruments). The clear supernatant was transferred to a fresh tube and used for RNA isolation. Chloroform was added (0.2 ml per ml of trizol used) for phase separation, mixed well and incubated for 15 min. at room temperature followed by centrifugation at 13 K at 4°C for 15 min. The aqueous phase was collected (taking care not to disturb the interphase) in a fresh tube and isopropanol (0.5 ml per 1 ml of Trizol) was added for precipitation of the RNA. The pellet obtained after centrifugation for 20 min. at 4°C was washed twice with 70% ethanol, air dried briefly and dissolved in 20-30 μl of autoclaved distilled water. The quality and quantity of RNA was estimated by separating 1 μl of RNA on a denaturing 1.2 % agarose gel (MOPS buffer). RNA was also quantified by spectrophotometry at wavelengths 260 and 280 nm.

Northern hybridisations

10-20 μg of total RNA isolated using Trizol (Gibco) was used for each northern blot. Separation of the denatured RNA on a 1.2 % denaturing agarose gel (1 X MOPS buffer), transfer of fractionated RNA to Hybond N nylon membrane (Amersham) by capillary method were performed according to Sambrook et al., 1989. The RNA was immobilized on the membrane by UV crosslinking, at 120,000 μJ/cm² energy setting on the Amersham UV crosslinker. For hybridisation and detection of the signal, procedure described earlier under Southern hybridisation was followed.

RT-PCR analysis

To determine the level of gene expression semi-quantitative RT-PCR analysis was used. In a RT-PCR the RNA is reverse transcribed to cDNA and the desired target cDNA can be amplified using specific primers. This is a highly sensitive technique as small changes in the expression can be detected.

Reverse Transcription:
Materials and Methods

RNA was isolated using Trizol (Invitrogen) as described above. To remove the genomic DNA contamination, the RNA from different samples was treated with RQ1, RNase-free DNase (1U, Promega), for 45 min at 37°C followed by inactivation of the enzyme at 75°C for 15 min. The samples were tested for the absence of DNA by performing PCR for GAPC on the RNA samples before proceeding for RT. The RT reactions were performed (with approx 1-2µg of RNA) using Superscript RT enzyme (Invitrogen) according to the manufacturer's instructions. For the first strand synthesis an Oligo dT (17mer) primer was used. Levels of GAPC were used as equalizing control while comparing multiple RT samples within one set of experiment.

Determining the linear range

The sensitivity of the RT-PCR depends on the exponential increase of the product. Theoretically the amount of PCR product doubles during each PCR cycle (a linear relation between the input and the output), but actually beyond a particular number of cycles the efficiency of amplification decreases resulting in a plateau effect (the input and output do not show a linear relationship). A number of factors like inactivation of DNA polymerase, degradation of nucleotides or primers, competition of non-specific DNA products, accumulation of inhibitors of polymerase etc. can contribute to this plateau effect. The number of PCR cycles at which the plateau effect occurs may vary for different DNA templates. The number of cycles, for each of the AML genes (AML1-AML4), AGL20 and GAPC at which a linear relationship occurs between the input and the output, was determined over a range of dilutions. Four to five serial dilutions of the leaf RT sample were made such that they span a range of approx. 200 folds dilution. The complete set of dilution series were then amplified for different number of cycles (15-29). Tubes for one entire set of dilution series were removed after the completion of 15, 19, 21, 23 and 26 cycles. 10µl of the PCR products were separated on a 1 % TAE agarose gel; the DNA was denatured and transferred onto Hybond N nylon membrane by vacuum transfer. PCR labelled internal probes (not including either of the primers used in the RT-PCR) were used for Southern hybridisations. The intensities of the hybridisation signals for different dilutions at each cycle were recorded and plotted on a log scale.
The cycles for which a linear relationship between the input and the output could be seen over a wide concentration range were selected for each of the genes (AML1-AML4, AGL20 and GAPC). In all the subsequent experiments these were used as a standard number of cycles representing the linear range for the respective genes. The linear range was separately determined for each RT reaction in this manner.

List of PCR primers used and PCR conditions used for the Semi-Quantitative RT-PCR analysis.

**AML1**: 517 bp

AML1 sac: TCA AGA GCT CAA TCG ACT GGT C
AML1 up: CAG AAA ATG CAG AGG ACG TAT ATG

- 93°C 0.20 sec
- 56°C 0.45 sec
- 72°C 1:00 min
- 22 cycles

**AML2**: 880 bp

AML2: 880 bp

- 93°C 0.20 sec
- 57°C 0.45 sec
- 72°C 1:00 min
- 22 cycles

**AML3**: 680 bp

AML3: 680 bp

- 93°C 0.20 sec
- 57°C 0.45 sec
- 72°C 1:00 min
- 23 cycles

**AML4**: 839 bp

AML4: 839 bp

- 93°C 0.20 sec
- 56°C 0.45 sec
- 72°C 1:00 min
- 22 cycles

**GAPC**: 541 bp

GAPC: 541 bp

- 93°C 0.20 sec
- 56°C 0.35 sec
- 72°C 1:00 min
- 18 cycles

**AGL20**: 540 bp

AGL20: 540 bp

- 93°C 0.20 sec
- 55°C 0.35 sec
- 72°C 1:00 min
- 21 cycles
**Probe preparations for Northern and Southern**

All probes used in this study were labelled either by random primer (RP) or PCR labelling. Random primer labelling was performed using the RP kit (BRIT). Template DNA (approx. 50 ng) was heat denatured at 100°C for 2 min and added to a reaction mix containing 0.8 mM of all the dNTPs except dATP, random primer and Klenow (2U). The reaction was incubated at 37°C for 1.5 hrs in the presence of 50 μCi (5μl) of αP32dATP (supplied by Jonaki). In some cases αP32 dCTP (NEN) was used for the labelling purposes.

For PCR labelling the PCR reaction was done in the presence of low conc. of all the four dNTPs (5 μM) and 2.0 μl of αP32dATP/ αP32dCTP. All other conditions were the same as for the cold PCR that had been optimised for that particular probe. In each case the PCR was carried out for 35 cycles for preparing the labelled probes. The primer pairs used for PCR labelling are listed in Table 6.3. The labelled probes were purified from the unincorporated nucleotides by gravity column (size exclusion chromatography) (Boigel P-60, Pharmacia). The purified probes were stored in −20°C and were heat denatured (100°C for 5 min.) before adding to the blots at a concentration of >10^6 cpm/ml of hybridisation solution.

**Expression Studies for the AML Genes**

RNA In-situ Hybridisations were used to study the cellular and spatial localization of the AML transcripts. We followed David Jackson’s and J-Ph. Viel-Calzada’s protocol for RNA in-situ hybridisations with slight modifications.
<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the plasmid</th>
<th>Gene</th>
<th>Internal Primer Pair Used</th>
<th>Size of the probe</th>
</tr>
</thead>
</table>
| 1     | pMOS-140J           | AML3 | 140jIU: ctg agg agt gtg  
gag cta taa acc tca  
140JID: tcc aca ggt tca ttg  
agt act atg atg          | 700   |
| 2     | pSP- 40B12          | AML2 | 40BIU: ctt ggt tgt ttt cat  
cga tgt cag cta  
40BID: cat gtt ggg tgt gca  
cca tct g          | 750   |
| 3     | pMOS-Chr4           | AML4 | Ch4IU: cct tgt gca agc tct  
cca ata gg  
Ch4ld: gga aac cat tgt  
agc agc agg c          | 700   |
| 4     | pGEM-AML1           | AML1 | AMLIU: atg gag att tgg  
cat gct tgt ga  
AMLID: cag cag ctc gtc  
ttg tgt gac ta          | 500   |
| 5     | pMOS-GAPC           | GAPC | GAPIF: gtt gtt ggt gtc aac  
gag cac gaa tac  
GAPIR: gag aca tca tcc  
tcg tgt tat cc          | 455   |
| 6     | pMOS -AGL20         | AGL20| AGLIF: tga gct ctc tca gtg  
cct tgt gat gct  
AGLIR: ctt ggg cta ctc tct  
tca tca cct ctt c          | 450   |
| 7     | pMOS – AP1          | AP1  | APIu: aac agg ctt aag gct  
aag att gag  
APId: ctt cca ttt gat cat  
ctt ctt g          | 415   |

Table 6.3  
List of plasmids and Primer Pairs used for making PCR labeled probe for the RT-PCR analysis
Fixing the tissue and preparing Blocks

Fresh tissues (inflorescence, leaves, seedlings, siliques) were collected for fixing. Large leaves were cut into 0.5 cm pieces and for siliques both the top and bottom ends were cut to enable efficient infiltration of the fixative. The tissues were put into tissue cassettes (Fisher Scientific) to prevent them from floating away. The cassettes were immersed in freshly prepared FAA (4 % freshly prepared PFA, 5% acetic acid, and 50 % ethanol) and vacuum was applied slowly for 30 min. This step was repeated (4-5 times) till the tissue sank down, ensuring proper infiltration of the fixative in the tissues. The tissue cassettes were transferred to a beaker containing fresh FAA and kept overnight at 4°C. After fixing the tissues were dehydrated immediately by processing them through an ethanol series (60, 70, 80, 90, 95, and 100 %) at room temperature. The samples were incubated in each of these ethanol concentrations for 30 minutes and finally left in 100 % ethanol for overnight. The tissues were removed from the cassettes and transferred into 10 ml glass vials and 100 % ethanol was replaced by 3:1 ethanol: xylene, followed by 1:1 ethanol: xylene, 1:3 ethanol: xylene and finally with pure xylene (1hr each). The samples were then shifted to 55°C and slowly over the next three days the xylene was completely replaced by 4 to 5 changes of paraffin wax (Tissue Prep 2; Fisher Scientific). The tissues were finally embedded in Tissue Prep 2 using Leica EG1140H embedder.

Sectioning

10-14 micron thick sections were cut using Leica RM 2135 microtome. The best sections were selected and put on to Probe On Plus positively charged slides (Fisher Scientific) and baked for 14 -16 hrs at 42°C on a slide warmer. These slides could be stored at 4°C for 1-2 weeks.

Ribo- Probe preparation

The plasmid (prepared using Qiagen tips) containing the template was linearised at the 3' end with the appropriate restriction enzyme. After complete linearization the restriction enzymes and other protein contaminations were removed by one round of phenol: chloroform extraction, followed by two rounds of chloroform extractions. The
DNA was precipitated, resuspended in a minimum volume of 2 mM Tris (pH8) and quantified by gel electrophoresis. 1 μg of the linearised DNA was used for each transcription reaction (performed according to the manufacturer's instructions). To confirm that the *in vitro* transcription had worked 0.5 μl of the reaction was checked on a 1.5 % TAE gel. The template DNA was degraded using RQ1, RNase free DNase (Promega). For better penetration the probes were alkali-hydrolysed to a final size of 100 bp. The time (t) for which the hydrolysis was carried out at 60° C was calculated according to the formula

\[ t = \frac{(L_i - L_f)}{k} \times L_i \times L_f \]

where \( t \) = time of hydrolysis in minutes; \( L_i \) = initial length (kb) ; \( L_f \) = final length (kb) ; \( k \) = rate constant (0.11 kb/min). To precipitate the hydrolysed products ammonium acetate to a final concentration of 0.5 M and 3 volumes of 100% ethanol were added and the tubes were incubated overnight at -70°C. The pellet obtained after centrifugation at 13 K at 4°C was resuspended in 50 μl of water, and 1 μl was checked on a 1.5% TAE gel for approximate quantitation.

<table>
<thead>
<tr>
<th>Name of the construct</th>
<th>Plasmid backbone</th>
<th>Size of the probe</th>
<th>Enzyme used for linearization</th>
<th>Probe</th>
<th>RNA pol used</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSP40-Xba</td>
<td>pSPORT</td>
<td>700bp</td>
<td>Xba1</td>
<td>Sense</td>
<td>T7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EcoR1</td>
<td>Antisense</td>
<td>SP6</td>
</tr>
<tr>
<td>pAML3 1.2</td>
<td>pSPORT</td>
<td>1200bp</td>
<td>Pvu1</td>
<td>Sense</td>
<td>T7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EcoR1</td>
<td>Antisense</td>
<td>SP6</td>
</tr>
</tbody>
</table>

Table 6.4
Plasmids used for ribo-probe synthesis for *in situ* hybridisations

*Pre-Hybridisation*

The sections were dewaxed by dipping the slides in Histoclear for 15 min. followed by incubation for another 15 min in fresh histoclear. The slides were then processed through an ethanol series (100 % X 2, 95, 85, 70, 50, and 30) at room
temperature to rehydrate the tissue, and finally rinsed in 1 X PBS. The tissue was permeabilized with Pronase type XIV (Sigma) at a final concentration of 0.125 mg/ml in 50 mM Tris (pH7.5); 5 mM EDTA at 37°C for 18 min for better probe penetration. To stop the pronase action the slides were immersed in 0.2 % glycine (in PBS) followed by refixing them in PFA (freshly prepared). To prevent the non-specific binding of the probe the sections were acetylated (using 0.1M triethanolamine-HCl (pH8) and acetic anhydride). The sections were dehydrated through the ethanol series from 30%, 50%, 70%, 85%, 95%, and 100% and air dried. While preparing the hybridisation sol. the slides were kept in an ethanol-saturated chamber and kept at 4°C.

**Hybridisation**

The quantity of probe to be used to achieve a detectable signal (~ 0.3ng/μl hyb. sol. per kb probe complexity) was determined for each batch of probe. The probe was added to 20 μl of 50% formamide solution, heat denatured at 80°C for 3 min and immediately kept on ice. To the probe / formamide mixture 160 μl of hybridisation solution was added and evenly applied to the slides. Hybrislip (Fisher) or a sterile Parafilm piece was used to cover the slide. The slides were placed in RNase free, airtight plastic boxes (humidified with 2 X SSC and 50% formamide), which were wrapped in saran and incubated at 55°C for at least 16 hrs.

**Post–hybridisation**

The slides were washed twice with 0.5 X SSC for 1 hr each at 55°C. The slides were then rinsed in NTE (0.5 M NaCl; 10 mM Tris (pH7.5); 1 mM EDTA) and treated with 10 μg/ml RNase at room temperature for 20 min. to reduce the background staining. Subsequently the slides were washed in NTE (5 min) at room temperature, 0.2 X SSC for 1 hr at 55°C. After rinsing the slides in 1X PBS for 10 min. the sections were blocked in 0.5% Boehringer block in TBS (100 mM Tris (pH7.5); 150 mM NaCl) for 45 min. at room temperature, followed by another wash for 30 min. The sections were finally blocked in 1% BSA; 0.3% TritonX-100 in TBS at room temperature for 45 min. Anti–Dig antibody (Roche) was diluted 1:1250 times in BSA block and applied to the sections for 2 hrs in a humidified chamber. This was followed by 4 washes in 1%
Materials and Methods

SA; 0.3%TritonX-100 in TBS and once in the detection buffer (Buffer C: 100 mM Tris pH9.5; 50 mM MgCl2; 100 mM NaCl). The color reagent (NBT+BCIP) (Roche) was diluted 1:50 times in Buffer C and added to the sections. The slides were incubated in a humidified chamber in dark and monitored regularly for the level of the signal developed. The color reaction was stopped by washing the slides in distilled water twice) and mounted in 50% glycerol. The sections were observed using Axioplan 2 microscope using the DIC optics and the pictures were captured on Kodak upra 100 film (ISO 100). The prints were scanned and processed in Adobe Photoshop Version 5.

Agrobacterium transformation (Triparental matings)

Triparental matings were done to mobilize the donor plasmid (binary vector with the insert of interest) from E. coli (DH5α) strain to Agrobacterium (AGL1) using an E. coli strain (HB101) carrying a helper plasmid pRK2013. 10 ml Agrobacterium culture was grown in TYM broth containing Carbenicillin (50 μg/ml) for 48 hours. 5 ml of the donor and the helper strains was grown overnight in LB containing respective antibiotics (Chloramphenicol 25 μg/ml and Kanamycin 50 μg/ml). Cells were pelleted and washed with sterile water to remove traces of antibiotics, and finally resuspended in 200 μl of LB each. The three strains (recipient, helper and donor) were mixed in 1:1:1 ratio of Agrobacterium: helper: donor, and spotted on autoclaved N+ HYBOND nylon membrane (Amersham), which were placed on LB plates. The triparental mix was incubated at 28°C for 12hrs. The bacterial cells were scraped off from the membrane and resuspended in 1 ml sterile LB. Serial dilutions were made and 100 μl of 10⁻¹, 10⁻³ and 10⁻⁶ dilutions were plated on TYM plates with appropriate antibiotics (Carbenicillin 50 μg/ml and Chloramphenicol 25 μg/ml) for selecting the exconjugants.

In planta transformation by vacuum infiltration

The Agrobacterium mediated in planta transformation by vacuum infiltration protocol used in this study was a modification of Bechtold et al., 1993. Small (square) pots were filled above the brim with the soil mix, soaked in Nutrient solution, covered with a nylon mesh and around 6-8 seeds were evenly spread on the top. After 2-3
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days of stratification the pots were shifted to the growth chamber and the plants were
grown under long day conditions for three weeks till they started to bolt. The primary
bolt was clipped to encourage secondary inflorescence to emerge. Any siliques that
were elongating prior to infiltration were trimmed.

*Agrobacterium tumefaciens* strain (AGL1) carrying the gene of interest on a
binary vector was grown in a large liquid culture at 28° C in TYM with required
antibiotics. The culture was allowed to reach the mid-log phase, OD 600 more than 0.8
(approx). The cells were then spun down and resuspended in the infiltration media
pH5.7 (IM: half strength MS medium supplemented with 10 μg/L 6-benzyl aminopurine,
and 5% sucrose). Before dipping the plants wetting agent SilwetL-77 (Osi Specialities
inc.) was added to the final conc. of 0.02%.

For vacuum infiltration the above ground parts of the plants were dipped by
inverting the pot over a 250 ml beaker containing the *Agrobacterium* solution and 500-
mm Hg vacuum was applied for 15 min. The vacuum was released rapidly. The pots
were placed horizontally (on their sides) in a tray, covered with saran wrap to maintain
humidity, and allowed to recover overnight in the growth chamber before keeping them
upright. A second round of infiltration for the same set of plants was done after an
interval of 4-5 days. T1 seeds were harvested in bulk and then selected on MS agar
plates + 2% sucrose containing Kanamycin (50 μg/ml). Kanamycin susceptible
seedlings turned yellow whereas the resistant seedlings remained green and produced
first pair of true leaves. These KanR plans were transplanted to soil. T2 seeds from
individual plant were collected for further analysis.

**Histology**

*Whole mount of Ovules and pollen*

The flowers or inflorescence were fixed in FAA (3.7 % formaldehyde, 5%
acetic acid, 50% ethanol) overnight at 4° C. Samples were rinsed in 50% acetone and
dehydrated in acetone series (60%, 70%, 80%, 90%, 95%, and 100%). For clearing,
the samples were incubated in methyl benzoate for 1 hr, followed by an overnight
incubation in methyl benzoate: Spurr's (7:1). The pistils and anthers were dissected on
clean glass slides, mounted with a cover slip and observed under DIC optics on
Axioplan imaging 2 microscope. The pictures were captured using a 40X oil immersion objective on either a slow speed (50 ISO) black and white film or on a CCD camera.

**GUS staining**

Tissues (seedlings, leaf, inflorescence, and siliques) were collected and transferred to a 24 well microtiter dish containing 0.5 ml to 1 ml GUS staining solution (100 mM Na Phosphate buffer (pH7), 10 mM EDTA, 0.1% Trition X-100, 1 mg/ml X-Gluc (Biosynth AG), 100 μg/ml Chloramphenicol, 1 mM potassium ferrocyanide and 1mM potassium ferricyanide) (Sunderasan et al., 1995). In case of ovules, the pistils were removed from the flowers, and the pistil wall was slit along its length before transferring to the staining solution. For staining the ovules the pistils were partially dissected to expose the ovules to the staining solution. The microtiter dish was placed under vacuum for 10 min to ensure proper infiltration of staining solution. After releasing the vacuum (very slowly) the dishes were covered with foil, and incubated at 37°C for 12-48 hrs. The expression was checked intermittently. At the end of the staining the tissue was cleared by incubating with 50%, 60% and finally 70 % ethanol at 37°C. For ovules the pistils were directly dissected in lactic acid: glycerol clearing solution and mounted in the same. Inflorescence, seedlings, siliques, and leaf samples were observed under Zeiss dissection microscope and images were captured on the digital camera. The ovule and pollen slides were observed under DIC optics on Axioplan imaging 2 microscope. The pictures were captured using a 20 X or 40 X oil immersion objectives on a CCD camera.

**Other Methods**

For all the other molecular methods that are not mentioned in this chapter, but were used in this study the protocols given in Sambrook et al., 1989 were followed.

**Programs used for DNA and Protein sequence analysis**

**BLAST**: Used for sequence comparison (Altschul et al., 1990)

**AMPLIFY 2.5β**: Used for designing PCR primers (William Engels, Genetics Department, University of Wisconsin)
Materials and Methods

**Gene Tool:** Used for analysis of DNA sequences (electrophorograms) and designing PCR primers (Wishart *et al.*, 2000)

**DNA strider:** Used for in silico restriction analysis of the DNA sequence (Ch Marck and CEA, 1991)

**Clustal:** Used for aligning different AML (AML1-5) protein sequences (Thompson *et al.*, 1997)

**Commonly used solutions**

**Murashige And Skoog media (1X):** CaCl$_2$ (4 mM); MgSO$_4$ (1.5 mM); KNO$_3$ (18.8 mM); NH$_4$NO$_3$ (20.6 mM); KH$_2$PO$_4$ (1.25 mM); Fe-EDTA (20 mM); Minor Salts (1 X).

**Nutrient Solution (1X):** KNO$_3$ (5 mM), KH$_2$PO$_4$ (2.5 mM), pH5.6; MgSO$_4$ (2 mM); Ca(NO$_3$)$_2$ (2 mM); Fe-EDTA (20 mM), and Minor Salts (1X), (Somerville and Ogren, 1982).

**TYM For 1 liter:** Bacto tryptone (5 g), yeast extract (0.5 g); mannitol (10 g); CaCl$_2$ (1 mM/ltr).

**20 X SSC:** 3 M NaCl; 0.3M Sodium Citrate, pH7.0.

**Denaturation Solution:** 1.5 M NaCl and 0.5 M NaOH.

**Depurination Solution:** 0.125 N HCl

**Hybridization Solution for in situ:** 50% formamide; 1X Denhardt's Sol; 10% Dextran Sulphate; salts (NaCl (300 mM); Tris pH7.5 (10 mM); NaPO$_4$ buffer (10mM); EDTA (1 mM); tRNA (150 µg/ml).

**Denhardt's solution (50X):** 1% Ficoll 400; 1% poly vinyl pyrrolidine; 1% BSA fraction V.

**Spurr's resin:** 4-Vinyl-cyclohexane-dioxide 22.8%(v/v), DER-736 resin 13.09%(v/v), 2-noenyl-succinic-anhydride 62.96%(v/v), Dimethyl Amino Ethanol 1.12% (v/v)
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Table 6.5 List of plasmids used in this study