Chapter Two

Materials and Methods

2.1 Plant growth conditions for flowering time experiments

Seed was surface sterilized by immersion in a solution of 5% sodium hypochlorite, containing 5 drops of Triton X-100 per 100 ml, for 5 minutes, followed by 5 rinses in sterile water. Each seed was placed into a glass test-tube containing 7 ml of growth medium containing 1X MS iron, macro- and microelements, 0.2X MS vitamins (Murashige and Skoog, 1962), 1.5% sucrose and 0.75% noble agar, pH 7.0. Seed were incubated at 4 °C in darkness for 72 hours to break dormancy, or for 21 days to vernalize the seedlings. After these treatments, the plants were transferred to growth cabinets at 21-23 °C, with light conditions as described in the following chapters.

2.2 Genomic DNA extractions

2.2.1 CsCl gradient method of DNA purification

For each sample, 5 g of tissue was ground to a fine powder in liquid nitrogen, transferred into 5 ml of pre-warmed extraction buffer (2% CTAB, 100 mM Tris (pH 8.0), 20 mM EDTA, 1.4 mM NaCl, 2% β-mercaptoethanol) and incubated at 65 °C for 5 minutes. After addition of 10 ml of 24:1 chloroform:isoamyl alcohol, the sample was shaken vigorously for 15 minutes and centrifuged for 15 minutes at 1700 x g. The aqueous phase

was transferred to a fresh tube and the chloroform:isoamyl alcohol extraction repeated. 2 volumes of precipitation buffer (1% CTAB, 50 mM Tris (pH 8.0), 10 mM EDTA, 1% β-mercaptoethanol) was added and the mixture was incubated at room temperature overnight. After centrifugation at 10 000 x g for 10 minutes the supernatant was discarded. The pellet was dissolved in 2 ml of 1M cesium chloride-TEN (1M CsCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 50 mM NaCl). 20 µl of 10 mg/ml ethidium bromide was added, and the mixture was overlaid on 2.5 ml of 5.7 M cesium chloride-TEN (5.7 M CsCl, 50 mM Tris (pH 8.0), 5 mM EDTA, 50 mM NaCl). The gradient was centrifuged at 36 000 rpm for 16-18 hours in a Beckman SW50.1 rotor at 20 °C, resulting in separation of genomic DNA into a band visible under UV light, which was collected by side puncture of the tube using an 18 G needle. The ethidium bromide was extracted by mixing with an equal volume of CsCl-saturated isopropanol and removal of the upper phase; this was repeated 3 times. The DNA was precipitated by the addition of 3 volumes of 70% ethanol. The DNA was either spooled on a glass pipette or pelleted by centrifugation at 700 x g for 15 minutes, dissolved in 500 µl of TE, and transferred to a 1.5 ml eppendorf tube. The DNA was re-precipitated by the addition of 50 µl of 3M sodium acetate (pH 5.2) and 1 ml of ethanol, and incubated at room temperature for 30 minutes. After centrifugation for 15 minutes at maximum speed in an eppendorf centrifuge at 4 °C, the supernatant was discarded and the pellet air dried. Depending on pellet size, the DNA was dissolved in 100-250 µl of TE. The DNA yield was estimated by measuring the absorbance at 260 nm.

A smaller scale preparation used 2 g of starting material, and all amounts were scaled down accordingly. Ultracentrifugation was performed for 2.5 hours at 55 000 rpm in a Beckman TES55 rotor at 20 °C.

2.2.2 Dellaporta DNA mini-preparation

The "Dellaporta" method for small scale plant DNA isolation is based on the method reported in Dellaporta *et al*. (1983), as modified by Ottoline Leyser and Mark Estelle (Department of Biology, Indiana University, Bloomington, U.S.A.). One gram of plant tissue was frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. The powder was transferred to a 30 ml tube without allowing it to thaw, and 15 ml of extraction buffer was added. The extraction buffer contained 100 mM Tris (pH 8.0), 50 mM EDTA (pH 8.0), 500 mM NaCl and 10 mM β-mercaptoethanol, which was added just before use. The ground powder and extraction buffer were mixed thoroughly after the addition of one ml of 20% SDS. The mixture was incubated at 65 °C for 10 minutes. Five ml of 5M potassium acetate was added, and the mixture shaken. The tube was incubated on ice for at least 20 minutes. The sample was centrifuged at 25 000 x g for 20 minutes after which the supernatant was filtered through "Miracloth" (available from Calbiochem Corporation, La Jolla, U.S.A.) into a fresh tube containing 10 ml of isopropanol. The supernatant and isopropanol were mixed and incubated at –20 °C for 30 minutes. The sample was centrifuged at 20 000 x g for 15 minutes. The supernatant was discarded and the tube drained by inversion on a paper towel. The pellet was dissolved in 0.7 ml of 50 mM Tris, 10 mM EDTA (pH 8.0), and transferred into a 1.5 ml eppendorf

tube. The sample was centrifuged in an eppendorf centrifuge at high speed for 10 minutes to pellet insoluble debris, and the supernatant was transferred to a fresh tube. A one-tenth volume of 3M sodium acetate (pH 5.2) and a seven-tenths volume of isopropanol was added. After mixing, the DNA was pelleted by a 5 minute centrifugation at high speed in an eppendorf centrifuge. The supernatant was discarded and the pellet washed in one ml of ice-cold 80% ethanol. The pellet was air-dried and dissolved in 100 µl of TE. In cases where little plant tissue was available, the method was scaled down by two- to ten-fold.

2.2.3 Modified Edwards DNA mini-preparation

The Edwards plant DNA mini-preparation, based on the method reported in Edwards *et al*. (1991), provides DNA suitable for PCR amplification. DNA can be obtained from as little as one small leaf from young seedlings, and all plant parts seem suitable for extraction. Starting material was ground in a 1.5 ml eppendorf tube using a plastic pestle for approximately 10 seconds, and 400 µl of extraction buffer (200 mM Tris-Cl (pH 7.5), 250 mM NaCl, 25 mM EDTA and 0.5 % SDS) was added. The tissue was ground briefly in the extraction buffer. The sample was vortexed for 5 seconds, and left at room temperature until further samples were ready. After centrifugation for one minute in an eppendorf centrifuge at high speed, 300 µl of the supernatant was transferred to a fresh 1.5 ml eppendorf tube. An equal volume of isopropanol was added and, after mixing, the sample was left at room temperature for 2 minutes and then centrifuged for a further 5

minutes at high speed in an eppendorf centrifuge. The supernatant was discarded, and after air drying for 10 to 15 minutes, the pellet was dissolved in 100 µl of TE.

2.2.4 Klimyuk DNA mini-preparation

This mini-preparation method is based on that of Klimyuk *et* al. (1993). A single leaf was collected into a 1.5 ml eppendorf tube containing 40 µl of 0.25 M sodium hydroxide, and incubated for 30 seconds in a bath of boiling water. After addition of 40 µl of 0.25 M hydrochloric acid and 20 µl of a solution containing 0.5 M Tris-HCl (pH 8.0) and 0.25% NP_{40}, the sample was returned to the boiling water bath for 2 minutes, and then stored at 4°C.

2.3 Plant genotyping procedures

2.3.1 Southern analysis

Restriction digests included 2 µg of DNA, 1 X universal restriction buffer (33 mM Tris acetate (pH 7.9), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol), 2 mM spermidine and 10-20 units of the restriction enzyme. The volume was made up to 50 µl with gelatin (100 µg/ml), and digests were incubated overnight at the appropriate temperature. Digests were electrophoresed on 1% agarose gels in 1 X TAE (Sambrook *et al.*, 1989), and, after a 5 minute depurination treatment of the gel in 0.1 N hydrochloric acid, the separated samples were transferred to Hybond N+ nylon

membranes (Amersham), using 0.4 N sodium hydroxide as a transfer buffer. After overnight transfer, the nylon membrane was washed for one minute in 5 X SSC (Sambrook *et al*., 1989).

Membranes were hybridized using Church buffer (0.5 M sodium phosphate buffer (pH 7.2), 7% SDS and 1mM EDTA). Before use, 200 µl of 6 mg/ml salmon sperm DNA (Sambrook *et al*., 1989) was incubated in a boiling water bath and added to the Church buffer. Filters were pre-hybridized at 65 °C for at least one hour, after which the radio-labelled probe, mixed with 200 µl of 6 mg/ml salmon sperm DNA and boiled for 5 minutes, was added. Hybridization at 65 °C was performed overnight. DNA probes for this and other hybridizations were radiolabelled with [α-^{32}P] dCTP (Amersham) using the Megaprime DNA labelling system (Amersham). Filters were washed twice for 15 minutes in a solution containing 40 mM sodium phosphate buffer (pH 7.2) and 1% SDS at 65 °C or at room temperature, depending on the desired stringency.

2.3.2 PCR analysis for presence of *Npt*II gene

The antisense construct contains the selectable *Npt*II gene (Finnegan *et al*., 1996), allowing the presence of the transgene to be detected by a polymerase chain reaction, using template DNA prepared from a single leaf (section 2.2.4; Klimyuk *et al*., 1993). The PCR reaction mix contained 1 µl DNA, 10 mM Tris (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1µM forward primer (GAG GCT ATT CGG CTA TGA), 1 µM reverse primer (ACT TCG CCC AAT AGC AG) and 1 unit Perkin-Elmer *Taq*

DNA polymerase. Cycling conditions were an initial cycle of 94 °C for 4 minutes, 50 °C for 30 seconds, 72 °C for 30 seconds, then 34 cycles of 94 °C for 10 seconds, 50 °C for 10 seconds, and 72 °C for 30 seconds. The expected size of the amplicon was 238 bp, allowing the reaction products to be resolved by electrophoresis on a 2% agarose gel.

2.3.3 PCR analysis for *nga8* and *nga111* microsatellite markers

The nga8 primer sequences were GAG GGC AAA TCT TTA TTT CGG and TGG CTT TCG TTT ATA AAC ATC C, while the nga111 primer sequences were CTC CAG TTG GAA GCT AAA GGG and TGT TTT TTA GGA CAA ATG GCG (Bell and Ecker, 1994). The PCR reactions included 10 mM Tris (pH 8.8), 50 mM KCl, 5 pmoles of each of either the nga8 or nga111 primers, 200μM of each dNTP, 2 mM $MgCl_2$, and 2 units of Perkin-Elmer *Taq* polymerase in a volume of 20 μl. Template DNA was prepared by either a modification of the Edwards method (Section 2.2.3) or the Klimyuk method (Section 2.2.4); in either case, 1 μl of the final resuspension was used in each reaction. The PCR cycles consisted of a single cycle of 95°C for 2 minutes, 55°C for 15 seconds and 72°C for 30 seconds, followed by 39 cycles of 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 seconds. Reaction products were separated on 8% acrylamide gels.

2.3.4 PCR for GAPB CAPS marker

The GAPB primer sequences were TCTGATCAGTTGCAGCTATG and GGCACTATGTTCAGTGCTG (Konieczny and Ausubel, 1993). The PCR reactions included 10 mM Tris (pH 8.8), 50 mM KCl, 1 μM of each primer, 125mM of each dideoxyribonucleotide, 1.5 mM $MgCl_2$, and 1.25 units of Perkin-Elmer *Taq* polymerase in a volume of 25 μl. Template DNA was prepared by a modification of the Edwards method (Section 2.2.3), and 4 μl of the final volume was used in each reaction. The PCR cycles consisted of a thirty cycles of 95°C for 30 seconds, 56 °C for seconds and 72°C for 3 minutes. Reaction products were digested with *Dde*I (New England Biolabs) and separated on 1% agarose gels.

2.4 Estimation of methyl-cytosine levels by thin layer chromatography

2.4.1 Sample preparation

This method of estimating cytosine methylation measures the ratio of methylated cytosine to unmethylated cytosines within *Taq*I sites. Cleavage with *Taq*I yields fragments of less than 500 bp on average, so this method scans the full genome. *Taq*I recognises the sequence TCGA, and cleaves 5' to the cytosine residue regardless of its methylation status.

One μg of DNA was digested with 10 units of *Taq*I, using the *Taq*I restriction buffer (New England Biolabs), in a final volume of 50 μl. Undigested samples were prepared in parallel, omitting only the *Taq*I. The digestion was performed at 65 °C overnight; samples were overlaid with mineral oil to prevent evaporation. Samples were transferred to a fresh 1.5 ml eppendorf tube using a thin pipette to avoid transferring mineral oil. Five μl of ribonuclease A at 10 mg/ml was added, and the samples were incubated for one hour at 37 °C and extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). After centrifuging at maximum speed in an eppendorf centrifuge for 15 minutes, the aqueous phase was transferred to a fresh tube. The DNA was precipitated by addition of a one-tenth volume of 3M sodium acetate (pH 5.2) and two volumes of ethanol followed by incubation at room temperature for 15 minutes, and pelleted by centrifugation in an eppendorf centrifuge at maximum speed for 15 mintes. The pellet was washed with 70% ethanol, re-centrifuged as above and air-dried. The pellet was dissolved in 50 μl of 1 X calf intestinal alkaline phosphatase (CIP) buffer (Gibco BRL) and incubated with 1 unit of CIAP (Gibco BRL) at 65 °C for 2 hours. 2 μl of 10 mg/ml proteinase K was added, and the sample was incubated at 37 °C for 2 hours. After extraction with phenol:chloroform:isoamyl alcohol (25:24:1) the DNA was precipitated with a one-tenth volume of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol at room temperature for 15 minutes, and centrifuged in an eppendorf centrifuge at maximum speed for 15 minutes. The pellet was air-dried and dissolved in 44 μl of water, to which was added 5 μl of 10 x polynucleotide kinase (PNK) buffer (MBI Fermentas), 1 μl of [γ-^{32}P] ATP (Amersham) and 10 units of PNK (MBI Fermentas). The sample was incubated at 37 °C for 90 minutes; another 10 units of PNK was added after 45 minutes. The

sample was passed through a 1 ml syringe packed with Sephadex ® G-50 (Pharmacia) by centrifugation at 1700 x g. The recovered sample was precipitated with a one-tenth volume of 3 M sodium acetate (pH 5.2) and 2 volumes of ethanol at –20 °C for at least one hour. After centrifugation at maximum speed in an eppendorf centrifuge for 15 minutes, the pellet was air dried and dissolved in 50 μl of TM (50 mM Tris (pH 8.0), 5 mM magnesium chloride) containing 10 μg of deoxyribonuclease I. The sample was incubated at 37 °C for at least 3 hours; 0.5 μg of phosphodiesterase I (type II; from *Crotalus adamanteus* venom; 5 mg/ml stock in 50 mM glycine-NaOH, 45% glycerol; Sigma catalogue number P6877), diluted 1 in 10 in TM, was added. The sample was incubated at 37 °C overnight, resulting in cleavage of DNA to deoxyribonucleotide monophosphates (dNMPs).

2.4.2 Thin layer chromatography

The dNMPs were separated on Kodak Chromagram thin layer chromatography (TLC) sheets which had been prepared by three runs with methanol and allowed to dry completely after each run. 2.5 μl of each sample was mixed with 2.5 μl of unlabelled dNMPs which included 5-methyl cytosine ($d^{m}CMP$), pipetted onto the TLC sheet and allowed to dry. The dNMPs were separated in a buffer consisting of 66 ml isobutyric acid, 20 ml water and 1.5 ml of 27% ammonia. After migration of the buffer front to the top of the plate, the sheet was dried and exposed on a Molecular Dynamics PhosphorImager cassette.

2.4.3 Analysis of thin layer chromatography

Radioactivity present in dCMP and d^{m}CMP was quantitated using a Molecular Dynamics PhosphorImager and IMAGEQUANT software. A wildtype sample was included on each chromatography plate for comparison with *METI* antisense lines. The uncut sample of each DNA sample was used to determine the amount of radioactivity incorporated due to shearing of the DNA; this background level was subtracted before calculating 5-methyl-cytosine levels by the formula d^{m}CMP/ d^{m}CMP + dCMP. 5-methylcytosine levels were normalised to wildtype controls.

2.5 Cloning and characterization of *METI* and *METII* genomic clones

2.5.1 Screening cosmid library

E.coli HB101 cells were grown overnight in a 37 °C shaking incubator in LB (Sambrook *et al*., 1989), harvested by centrifugation and resuspended in 4 ml of 10 mM magnesium sulphate. An aliquot of the cosmid library was added to 60 µl of HB101 cells and incubated for 10 minutes at 37 °C. After the addition of 1 ml of LB, the culture was shaken at 37 °C for one hour, harvested by centrifugation and the pellet spread on LB plates supplemented with 5 µg/ml tetracycline. The plates were incubated at 37 °C overnight. Hybond-N nylon filters (Amersham) were briefly laid on the plates, and then transferred to fresh LB plates supplemented with tetracycline, which were incubated overnight at 37 °C. The filters were placed on absorbent paper soaked in a series of

solutions: 10% SDS for 5 minutes, denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 5 minutes, and neutralizing buffer (1 M ammonium acetate, 20 mM NaOH) for 5 minutes; in between these steps the filters were briefly blotted on absorbent paper. DNA was immobilized onto the filter by UV cross-linking. Filters were hybridized as described in Section 2.3.1.

2.5.2 Cloning and hybridization procedures

Cloning was performed according to standard techniques given in Sambrook *et al*. (1989). Southerns of cosmid, lambda or plasmid DNA were performed using Hybond N+ membranes (Amersham) and Church buffer, as described for genomic Southerns in Section 2.3.1. Colony hybridizations were performed by inoculating two LB plates, one covered with a Hybond N membrane, with bacterial colonies and incubating at 37 °C overnight. Membranes were placed on absorbent paper soaked in a series of solutions: 10% SDS for 5 minutes, denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 5 minutes, and neutralizing buffer (1 M ammonium acetate, 20 mM NaOH) for 5 minutes; in between these steps the filters were briefly blotted on absorbent paper. DNA was immobilized onto the filter by UV cross-linking. Membranes were hybridized in Church buffer as described for genomic Southerns in Section 2.3.1. Plasmid DNA extractions were performed using an alkaline lysis procedure described in Sambrook *et al*. (1989) or using commercially available kits such as the Plasmid Midi Kit (Qiagen).

2.5.3 PCR for *METI* introns

The position of introns in *METI* was predicted based on sequence comparison of *METI* cDNA and *METII* genomic sequence, and primers were designed to flank the predicted introns (Table 2.1). Conditions for PCR were 10ng Arabidopsis ecotype C24 DNA, approximately 50 ng *METI* genomic λ clone or 1ng Yc8 *METI* cDNA clone in pUC119, 1μM each primer, 2mM $MgCl_2$, 200 μM each dNTP, 50mM KCl, 10mM Tris HCl pH 8.3 and 1 unit *AmpliTaq*® DNA polymerase (Perkin Elmer) in a final volume of 10 μl. Cycling conditions for PCR included an initial cycle of 95°C for 2 minutes, 46°C for 30 seconds, and 71°C for 30 seconds, followed by 25 cycles of 93°C for 30 seconds, 46°C for 10 seconds, and 71°C for 10 seconds. The final cycle was 93°C for 30 seconds, 46 °C for 10 seconds, and 71°C for 5 minutes. Reaction products were separated on a 2% agarose gel; products from the λ genomic clone were digested with *Eco*RI and cloned into pBSAmpSK for sequencing.

2.5.4 Sequencing

DNA sequences were obtained using *Taq* dye primer and dye terminator cycle sequencing kits (Applied Biosystems). Sequencing reactions were electrophoresed on the ABI Prism DNA sequencer Model 377, and analysed using ABI Prism DNA Sequencing Software Version 2.1.2. The λ and cosmid clones were subcloned into plasmid vectors to allow sequencing; *Exo*III nested deletions were generated to complete sequencing of some subclones, while for others, oligonucleotide primers were designed based on regions which had already been sequenced. Sequences were obtained for both strands of

Introns	5’	3’
II	TCAGAA**TTC**CTCAGTCAAATTAAA	GTCGAA**TTC**CACCAAGGGGCATAA
III-IV	GCGAATT**C**CTATGCTTCAGACAT	GCGAA**TTC**ATATTGGCGGGCAGC
V-VI	GCGAAT**TC**ATGGACTGAAAAAGG	GCGAAT**TC**CACCTTTCTCCATTA
VII-VIII	GCGAA**TTC**ATCAATGGTGGACCT	GCGAAT**TC**CAGGATTCCAAATCT
IX-X	GCGAA**TTC**CATCAGTAGAAAACG	GCGAAT**TC**AGATAACGTAACCTT
XI	GAATTC**CAGCCCATGGGTAAG**	GAATTC**GGGTTGGTGTTGAGG**

Table 2.1: Primers flanking putative introns in *METI*, predicted on the basis of comparisons of the cDNA sequence of *METI* with the genomic sequence of *METII*. Each primer contains an *Eco*RI site for ease of cloning, and the region of each primer which hybridizes to *METI* is shown in bold.

all subclones, and for all but one case overlapping clones were sequenced to confirm contiguity.

2.6 RNA extractions and expression studies

2.6.1 Total RNA extractions

One gram of tissue was ground to a fine powder in liquid nitrogen and transferred to plastic tubes. 2 ml of extraction buffer (8 M guanidine hydrochloride, 20 mM 2-(N-morpholino)-ethanesulfonic acid (pH 7.0), 20 mM EDTA, 50 mM β-mercaptoethanol) was added to the powder, followed by 3 ml of phenol:chloroform:isoamyl alcohol (25:24:1). The samples were shaken vigorously, and centrifuged at 1700 x g at room temperature. The aqueous phase was transferred to a fresh tube containing 3 ml of phenol:chloroform:isoamyl alcohol (25:24:1), and the extraction repeated. The aqueous phase was transferred to a 15 ml Corex centrifuge tube containing 600 µl of 1 M acetic acid and 2.1 ml of ethanol, mixed by inversion and incubated at –20 °C overnight. The samples were centrifuged for 25 minutes at 14 000 x g and the supernatant was discarded. The pellet was dissolved in 1 ml of sterile water and transferred to a 1.5 ml eppendorf tube. 220 µl of 10 M lithium chloride was added. The sample was mixed by inversion and incubated overnight at 4 °C. The sample was centrifuged for 15 minutes at maximum speed in an eppendorf centrifuge at 4 °C. After the supernatant was discarded, the pellet was dissolved in 500 µl of sterile water. The RNA was precipitated by addition of 50 µl 3M sodium acetate (pH 5.2) and 1 ml ethanol and incubation at –20 °C overnight. After

centrifugation for 15 minutes at maximum speed in an eppendorf centrifuge at 4 °C, the supernatant was discarded and the pellet was dissolved in 500 µl of sterile water. The RNA was quantified by measuring the absorbance at 260 nm, re-precipitated as described above, dissolved in sterile water to a final concentration of 5 µg/µl and stored at –80 °C.

2.6.2 Screening expression libraries

Four cDNA libraries were screened for evidence of *METII* expression. Library 1 was obtained from Dr Thomas Newman, of Michigan State University. The cDNA was derived from equal amounts of mRNA isolated from tissue cultured roots, 7 day old etiolated seedlings, and rosettes and aerial tissue (stems, flowers, siliques) from plants of different ages and 2 light regimes. All plants were ecotype Columbia. The library was cloned into a derivative of λZipLox, from which a plasmid containing the cDNA insert and M13 sequencing primer sites can be excised *in vitro* (D'Alessio *et al*., 1992). Library 2 was obtained from Dr Stephen Elledge, Baylor College of Medicine, Houston. The cDNA was derived from mRNA isolated from the above ground tissue of Arabidopsis plants ranging from plants with newly expanded primary leaves through to flowering plants. Plants were ecotype Columbia, with possible slight contaminations of Landsberg *erecta*. The library was made in λYES (Elledge *et al*., 1991). For sequencing, cDNAs isolated from this library were subcloned into plasmids containing M13 sequence primer sites. Library 3 was obtained from Dr Shiping Wang, of the Arabidopsis Biological Resource Centre at Ohio State University; it was made by Drs Detlef Weigel and Elliot Meyerowitz, of Caltech. The mRNA source was Landsberg *erecta* inflorescences with

flower buds predominantly younger than stage 11 (Smyth *et al*., 1990). The cDNAs were cloned into λZAPII. Library 4 was made by Dr Carol Grossman, of CSIRO Plant Industry, from apices of vernalized and unvernalized Landsberg *erecta* plants.

E. coli plating strains used were Y1090ZL for Library 1 and K803 for the remaining libraries. Cells were grown in Luria Broth (LB) to an optical density (at 600 nm; OD_{600}) of 0.7, collected by centrifugation and resuspended in 0.7 ml of 10 mM magnesium sulphate. 100 µl of the phage suspension was added to 10 µl of cells and incubated at 37 °C for 15 minutes. After the addition of 4 ml of λ-LB (LB plus 10 mM $MgSO_4$) containing 7 g/l agar, the mixture was poured over the surface of λ-LB plates containing 15 g/l agar, and incubated overnight at 37 °C. Hybond-N nylon membranes (Amersham) were briefly laid on the plates and removed. The membranes were treated for 10 minutes in denaturing buffer (1.5 M NaCl, 0.5 M NaOH), followed by 10 minutes in neutralizing buffer (1 M ammonium acetate, 20 mM NaOH), and DNA was immobilized onto the membranes by UV cross-linking. Membranes were hybridized in Church buffer as described for genomic Southerns in Section 2.3.1.

After exposure on autoradiograph film, plaques hybridizing to the probe were identified by alignment of the film, membrane and plate. Positive plaques were transferred into 500 µl of phage storage buffer (10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 10 mM magnesium chloride, 0.5 mg/ml gelatin), and re-plated and re-screened as above to confirm hybridization and to isolate single plaques to ensure purity.

Isolation of phage DNA was performed as follows. 1-10 plaques were added to 20 ml of NZCYM medium (Sambrook *et al*., 1989). After at least one hour at room temperature, 100 μl of plating cells, freshly prepared as above, were added. The culture was incubated at 37 °C for 15 minutes stationary, and then shaking overnight. Bacterial debris was pelleted by centrifugation at 10 000 rpm in a Beckman JA20 rotor for 10 minutes. The supernatant was centrifuged for 2 hours in a Beckman Ti60 rotor at 30 000 rpm. The pellet was resuspended in 300 μl PSB, to which 600 μg each of ribonuclease A and deoxyribonuclease I was added. After incubation on ice for 30 minutes, the sample was extracted with 600 μl of phenol:chloroform:isoamyl alcohol (25:24:1) three times. A one-tenth volume of 3M sodium acetate (pH 5.2) and 2 volumes of ethanol were added and the sample was incubated at –20 °C for at least one hour. The DNA was pelleted by centrifugation at maximum speed in an eppendorf centrifuge for 15 minutes. After washing with 70% ethanol and re-centrifugation as above, the pellet was dried and dissolved in 50 μl of TE.

2.6.3 Screening expression libraries by PCR for *METII*

Primers used to screen for *METII* expression were CTT CAT ATT CGC CGG AAA CTG C and AGC TAG TAA GGC TTC ATT TCA G. The product size from the predicted *METII* cDNA is 114 bp, while the product size from genomic DNA is 226 bp. The final reaction mix contained 10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 unit of Perkin Elmer *Taq* DNA polymerase, 2.5 μM of each primer, and an aliquot of the cDNA library containing 10^4 phage for library 1 and $3x10^3$ phage for

library 2. The positive controls contained either 1 ng of the 1.2 kb HindIII-HindIII fragment of Ac71, which contains intron II, or 100 phage of Ac71. The negative control contained 1 ng of the Y8 clone of the *METI* cDNA, which spans the region predicted to contain *METI* intron II (Genger *et al*., 1999). Cycling conditions were 95°C for 2 minutes, 42°C for 30 seconds, and 71°C for 1 minute, followed by 25 cycles of 93°C for 30 seconds, 42°C for 30 seconds, and 71 °C for 1 minute. The final steps were 42°C for 30 seconds, and 71°C for 5 minutes. Reaction products were electrophoresed through 4% acrylamide gels and visualised under UV light.

2.6.4 RT-PCR for *METII* and *METI*

Primers for reverse transcription were GCG AAT TCA TAT TGG CGG GCA GC and CTT CAT ATT CGC CGG AAA CTG C for *METI* and *METII* respectively. For each reaction, 100 ng of primer was annealed to 2 μg of total RNA by 5 minutes incubation at 65°C followed by cooling to room temperature. Final reverse transcription conditions were 10 mM $MgCl_2$, 10mM DTT, 50 mM Tris HCl pH8.3, 7.5 mM KCl, 0.5 mM dNTPs, 4 units Promega rRNasin® Ribonuclease inhibitor and 1 unit Epicentre Technologies MMLV reverse transcriptase, in a final volume of 10 μl. The reaction was carried out at 37°C for 60 minutes.

Primers for PCR on the reverse transcription reactions were GCG AAT TCC TAT GCT TCA GAC AT and AGC TAG TAA GGC TTC ATT TCA G for *METI* and *METII* respectively; these primers were chosen to span introns III and IV, providing a size

differential between products amplified from genomic DNA contaminants in the RNA samples and products amplified from reverse transcribed RNA. Half of each reverse transcription reaction was used for PCR. Final PCR conditions were 0.125 mM dNTPs, 50 ng of each primer, 2.5 mM $MgCl_2$, 2.5 mM DTT, 20 mM Tris-HCl pH8.3, 40 mM KCl and 1.5 units *Taq* DNA polymerase, in a final volume of 20 µl. Cycling conditions for *METI* PCR included an initial cycle of 95°C for 3 minutes, 48°C for 1 minute, and 72°C for 1 minute, followed by 25 cycles of 93°C for 30 seconds, 48°C for 30 seconds, and 72°C for 30 seconds. The final step was 72°C for 5 minutes. Cycling conditions for *METII* were identical, except that the annealing temperature was 58°C and 35 cycles were performed. Test reactions using serially diluted templates indicated that the sensitivity of *METII* PCR was roughly 1000 fold higher than that of *METI* PCR.

Fifteen µl of each RT-PCR reaction was electrophoresed on a 2% agarose gel and transferred to nylon filters after washing in denaturing buffer (1.5M NaCl, 0.5M NaOH) and neutralizing buffer (1M ammonium acetate, 20 mM NaOH). Filters were incubated at 65°C overnight in Church buffer as described in Section 2.3.1, with ^{32}P-labelled probes. Probes were purified from PCR amplification of the *METI* cDNA (Finnegan and Dennis, 1993) or the *METII* Ac71 genomic clone using the appropriate primers. Filters were exposed on Molecular Dynamics PhophorImager cassettes, and the intensity of RT-PCR products was quantitated using ImageQuant Software Version 3.3.

2.7 Plant transformation by vacuum infiltration

2.7.1 Cloning the *METI* antisense construct into a binary vector

The vacuum infiltration method (Bechtold *et al*., 1993) produces large numbers of seed which must be screened for potential transformants, and therefore selection with a herbicide provides a less labour-intensive screening method. For this reason, the *METI* antisense construct was cloned into a binary vector that contains the BAR gene for resistance to Basta® (active ingredient ammonium glufosinate). This vector, pMLBART, which carries a gene conferring resistance to streptomycin, was the gift of Michael Graham, CSIRO Plant Industry. pMLBART contains a unique *Not*I site between the left and right border, into which the *METI* antisense construct was cloned. However, it was first necessary to introduce a *Not*I site into the plasmid containing the *METI* antisense construct. Standard recombinant DNA protocols were used, as described in Sambrook *et al*. (1989).

The *METI* antisense construct, a 2.8 kb region of *METI* inserted between the cauliflower mosaic virus 35S promoter and the nopaline synthase 3' termination signal in the plasmid pJ35SN (Finnegan *et al*., 1996), was provided by E.J. Finnegan, CSIRO Plant Industry. The pJ35SN plasmid, which carries a gene conferring resistance to ampicillin, contains a *Sal*I site 5' to the 35S promoter; a *Not*I site was inserted into this *Sal*I site as follows. The pJ35SN-*METI* antisense plasmid was linearised with *Sal*I, and adapters complementary to the *Sal*I ends and containing a *Not*I site were ligated to the ends of the linearised plasmid.

The adapter is shown below, with the *Not*I site in bold:

TCGAC**GCGGCCGC**AG
||||||||||
G**CGCCGGCG**TC

After ligation, the adapters were cleaved with *Not*I and religated to form a new plasmid containing a *Not*I site between two *Sal*I sites. This plasmid and the binary vector pMLBART were cleaved with *Not*I and ligated. The resulting plasmid was introduced into the super-virulent *Agrobacterium tumefaciens* strain AGL1 (Lazo *et al*., 1991) using pRK2013 as a helper strain in a triparental mating.

2.7.2 Vacuum infiltration

The vacuum infiltration method used was a modification of the method of Bechtold *et al*. (1993). Soil grown Arabidopsis plants were grown until the primary bolt began to elongate, when it was excised to encourage development of secondary bolts. Infiltration was performed when these bolts were 2-5 cm tall. Plants were watered well a few hours before infiltration.

Two days before infiltration, a 25 ml culture of LB supplemented with 100 μg/ml ampicillin, 100 μg/ml streptomycin and 50 μg/ml rifampicin was inoculated with a single colony of *Agrobacterium tumefaciens* transformed with the *METI* antisense-containing plasmid described in Section 2.7.1. This culture was grown overnight in a shaking incubator at 28 °C, and used to inoculate a one litre LB culture supplemented with 100 μg/ml ampicillin, 100 μg/ml streptomycin and 50 μg/ml rifampicin. This culture was

grown until the OD_{600} was 0.8. The culture was centrifuged to collect the cells, and resuspended in 330 ml of infiltration medium (IM; MS medium plus 10 μg/l 6-benzylaminopurine and 5% sucrose; Murashige and Skoog, 1962).

Plants were uprooted, rinsed with water and immersed in IM, after which the IM and plants were placed under a vacuum of 75 mmHg for 20 minutes. Plants were then replanted and covered with plastic wrap which was removed after two days. Plants were allowed to set seed, which was harvested in bulk.

A number of variations to the above method which were tested in combination, are listed below. The one litre Agrobacterium culture was grown to an OD_{600} of 2, centrifuged and resuspended in a 3-fold volume of IM. Four alternate vacuum strengths were trialed: 100, 400, 500 and 625 mmHg; and shorter infiltration times of 10 or 15 minutes were tested. In some trials, the wetting agent Silwet L-77 (Osi Specialties, Inc.) was added to a final concentration of 250 μl/l. Another variation was to grow plants through a plastic mesh and invert the entire pot into the IM, to avoid the stress to the plants of uprooting and replanting.

2.7.3 Selection for transformants

Two selection methods, one involving soil grown plants and the second involving plants on growth medium, were used. Varying levels of herbicide were tested to determine the appropriate level for selection. Plants transformed with a construct encoding the BAR

gene for glufosinate resistance (gift of Ming Luo, CSIRO Plant Industry) were used as positive controls in these tests.

For selection of soil grown plants, seeds were sown into flats of river sand and sub-irrigated daily with 5 mg/ml of ammonium glufosinate, the active ingredient of Basta®. After 3 days, transformed (resistant) seedlings were green, while untransformed seedlings were yellowed and smaller than the resistant seedlings. For the second selection method, seed were surface sterilized as described in Section 2.1 and plated on MS plates containing 20 mg/l ammonium glufosinate. Under these selection conditions, transformed seedlings were mostly green, though some were yellowed and slightly smaller. All untransformed seedlings died after germination.