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# A novel *myb*-related gene from *Arabidopsis thaliana*

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**Abstract A novel *myb*-like gene (*Atmyb5*) has been isolated from a genomic library of *Arabidopsis thaliana*. The gene contains a single intron in the region coding for the Myb domains. The Myb domains are highly homologous to other animal and plant Myb proteins. *Arabidopsis* plants transgenic for a chimeric *Atmyb5* promoter/*GUS* gene expressed the enzyme in a developmentally controlled and tissue specific manner. The GUS activity was detected in developing leaf trichomes, stipules, epidermal cells on the margins of young rosette and cauline leaves, and in immature seeds. *Atmyb5* mRNA appears between fertilization and the 16 cell stage of embryo development and persists beyond the heart stage.**

*Key words: Arabidopsis thaliana*; *myb* gene; Seed development; Trichome; Stipule; Leaf margin

## 1. Introduction

Many of the genes controlling plant morphogenesis contain structural motifs characteristic of yeast and animal transcription factors [1]. For example, one of the genes controlling trichome formation in *Arabidopsis, Glabrous*-1 (*GL1*), encodes a Myb-related protein [2]. Many *myb*-like genes have been isolated from plants but the functions of only a few are known. In maize, three *myb*-like genes (*C*1, *P*1 and *P*) are required for the formation of flavonoids and derived pigments [3–5]. Another *myb*-like gene, *mixta*, affects the intensity of pigmentation of epidermal cells in the petals of *Antirrhinum majus* [6]. The cells of corolla lobes fail to differentiate into their normal conical form in the *mixta* mutants. Additional *myb*-like genes have been isolated from barley [7], maize [7], *Antirrhinum* [8], *Arabidopsis* [2,9,10], *Petunia* [11] and *Physcomitrella* [12].

The proteins encoded by these plant *myb*-like genes contain an N-terminal region which is highly homologous to the DNA-binding domains (Myb domain) of animal Myb proteins. However, while the animal Myb domains contain three imperfect repeats, the plant Myb domains contain only two. The animal and several plant Myb proteins have been shown to bind DNA in a sequence-specific manner [13–18]. Some of these Myb proteins have been demonstrated to act as transcriptional regulators for their target genes. The avian *mim*-1 gene is such a target gene regulated by the animal c-Myb protein [19]. In maize, *P* gene product activates the *A*1 promoter [20] whereas C1 protein plus another transcriptional regulator (R or B) is required for the activation of both *A*1 and *Bz*1 promoters [20–24].

This paper describes a novel *myb*-related gene, *Atmyb5*, we have isolated from *Arabidopsis thaliana* and presents studies on its expression pattern.

## 2. Materials and methods

A degenerate 38-mer oligonucleotide (CCTGGTCGTACTGA(C/T)AA(C/T)GA(A/G)ATTAA(A/G)AA(C/T)TA(C/T)TGGAA) covering the conserved region of the third repeat of the Myb DNA binding domains (see Fig. 3) was used to screen a genomic library of *Arabidopsis thaliana* (Landsberg *erecta*) in the vector EMBL3 according to the procedure described by Ausubel et al. [25]. Plaque hybridization with the $^{32}$P-labeled 38-mer was performed at 40°C and washing with $6 \times$ SSC (sodium chloride and sodium citrate) at 50°C. One of the positive clones identified was chosen for further study. To locate the region hybridizing to the 38-mer in the inserts, the DNA was subjected to single and double digestion with restriction enzymes *Bam*HI, *Eco*RI and *Sal*I and probed with the $^{32}$P-labeled 38-mer in a Southern hybridization. A 2.5 kb *Bam*HI–*Sal*I fragment was identified and subcloned into the vector pTZ18U (Fig. 1). The *Sal*I site was derived from the $\lambda$ vector. The fragment was further subcloned with the restriction enzymes shown in Fig. 1 and sequenced using both the sequenase method (U.S. Biochemical Corp.) and the dye-primer method with a DNA sequencer (Pharmacia). However, only part of *Atmyb5* gene was contained in the original insert. To clone the remaining portion of *Atmyb5*, *Arabidopsis* genomic DNA was digested with *Hin*dIII and these linear fragments were circularized by self-ligation. The mixture was subjected to the polymerase chain reaction (PCR) using a pair of primers hybridizing to *Atmyb5* (Fig. 2, coordinates +946 to +965, sense and +917 to +939, antisense). The PCR was carried out using the following conditions: 94°C 60 s, 58°C 45 s, 72°C 90 s, 1 cycle; 94°C 30 s, 58°C 30 s, 72°C 60 s, 41 cycles; 72°C 10 min. The amplified product of about 750 bp was cloned into pTZ19U using the *Spe*I and *Xba*I sites engineered in the primers and sequenced.

The *Bam*HI–*Bgl*II promoter fragment of *Atmyb5* (Fig. 1) was inserted into the *Bam*HI site of the plasmid pBI101.2 (Clontech) to create an *Atmyb5* promoter/*GUS* fusion. This *Atmyb5* promoter/*GUS* fusion plasmid should express a fusion protein containing the first 87 amino acids of Atmyb5 followed by the GUS protein sequence. The plasmid *Atmyb5* promoter/*GUS* was transformed into *Agrobacterium tumefaciens* strain AGL1 [26,27]. Root explants of *Arabidopsis thaliana* (Landsberg *erecta*) were transformed with the *Agrobacterium* using the method described by Valvekens et al. [28]. $T_0$ and $T_1$ plants of five independent transformants were obtained and subjected to detailed analysis. The incorporation of the *Atmyb5* promoter/*GUS* plasmid into the genome was confirmed by PCR amplification of the promoter insert from the genomic DNA with a pair of primers hybridizing to the vector sequences (5′-TGTGGAATTGTGAGCGGATA-3′ from +725 to +744 and 5′-ATTCCACAGTTTTCGCGATC-3′ from +909 to +929 bp of pBI101.2). The PCR conditions are described above. The amplified products were then analysed using restriction enzyme digestion.

Poly $A^+$ RNA was prepared by first collecting polysomal poly $A^+$ RNA and subsequently purified using Poly A Sepharose [29]. The RNA was electrophoresed on a 1.2% MOPS/formaldehyde gel [30] and blotted onto a Zeta probe in 50 mM NaOH as per manufacturer's instructions (BioRad). A Northern hybridization was carried out using a 3′ fragment of *Atmyb5* (from +946 to +1290, Fig. 2) labelled with $^{32}$P according to the standard procedure [25]. In brief, the hybridization was performed in formamide hybridization solution [25] at 42°C over-

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night and washed with 0.2 × SSC/0.1% SDS solution at room temperature. Histochemical GUS assays were carried out as described by Jefferson [31].

## 3. Results

The sequence of the *Atmyb5* gene, its 5′ upstream region and the deduced amino acid sequence of Atmyb5 are shown in Fig. 2. The region coding for about thirty amino acids at the carboxyl terminus and the 3′ untranslated region in *Atmyb5* (downstream from +1107 bp) was not included in the original $\lambda$ clone and was obtained by PCR (section 2). Five clones derived from the PCR fragments were sequenced. They were identical in sequence except for one base at +1120 (Fig. 2). Three of the clones contained an ATT codon which is translated into an isoleucine (Fig. 2B) and two contained an ACT codon which results in a threonine. Thus it appears that the two sequences may represent two alleles of *Atmyb5*. The N-terminal amino acid sequence of Atmyb5 is highly homologous to the Myb domains [13–18] and consists of two imperfect repeats, namely R2 and R3 (Fig. 3). The highly conserved tryptophan residues in c-Myb are also found in the Myb-like domains of Atmyb5. The one exception occurs at the beginning of R3 where the tryptophan is replaced by an isoleucine residue. The Myb domain of Atmyb5 exhibits a greater similarity to other plant Mybs (65% identity to GL1 and 68% to C1) than to animal c-Myb (46%). A short sequence of twenty amino acids at the N-terminus of Atmyb5 (from +1 to +65 bp) contains two serine and three threonine residues, which are potential protein kinase phosphorylation sites. The sequence at the carboxyl terminus of Atmyb5 possesses no significant homology with any of the other Mybs so far identified. However, two short sequences within the carboxyl region (Fig. 2) are identical or similar to sequences found in several other plant Myb proteins. The sequence GIDPQTHK in Atmyb5 is conserved in *Antirrhinum* Myb308, 315 and 330, barley Myb Hv1 and Hv33, and maize Myb Zm38 [7,8]. The sequence SSSHTDDTTVN is also conserved in *Antirrhinum* Myb330. The predicted molecular masses of Atmyb5 is 27.8 kDa (249 amino acids) and the gene contains a single 455 bp intron. Putative polyadenylation signal sequences were found at the 3′ ends of the gene (Fig. 2).

Plants transgenic for chimeric *Atmyb5* promoter/*GUS* genes were used to ascertain patterns of expression. Plant tissues at various stages of growth were stained for GUS activity. GUS activity (blue precipitate) was detected in developing trichomes, stipules, epidermal cells on the margins of growing rosette and cauline leaves, and immature seeds (Fig. 4). The GUS activity was initially detected in the first pair of trichomes on the first pair of leaves (Fig. 4A). The young leaf trichomes expressed relatively high activity (Fig. 4B,C) and as the trichomes became more mature, activity decreased (Fig. 4D). Low levels of GUS activity were detected in some trichomes of stems and sepals. GUS expression was detected at the margins of young leaves (Fig. 4C) and in stipules (Fig. 4B). GUS activity was also evident in young siliques (seed pods) (Fig. 4E). Examination of the sections of young siliques revealed that GUS activity was primarily expressed in the immature seeds and to a lesser degree in the silique epidermal cells (Fig. 4F,G,H,I). The GUS expression was detected in the majority of cells in the stained seeds.

Northern blotting was carried out to ascertain the time in seed development when *Atmyb5* mRNA is first detectable (Fig. 5). *Atmyb5* mRNA appears in the early stages of seed development, i.e. between fertilization and the 16 cell stage of the embryo. A very weak signal is detected in the pre-pollination buds sample. *Atmyb5* mRNA was present beyond the heart stage of embryo development, however whether levels are maintained through the fully formed seed remains to be determined. The approximate size of the mRNA band was 1.3 kb. The smearing of the bands might reflect a rapid turnover of

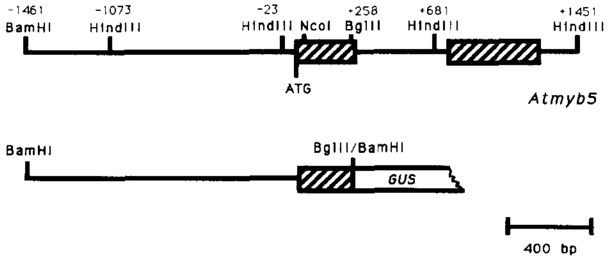

Fig. 1. Schematic diagram of *Atmyb5* and *Atmyb5* promoter/*GUS* fusion structures. Exons are represented by shaded boxes. Restriction sites used for subcloning and sequencing are shown.

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-1461                                     GGATCCCAACTCTGTAAACAGTACAAAATTC
-1430    AGAAAACTACCTTCAAAAGATCAATTTCTTGCTGGAGAAATTCATCCCAACAGCACAAGAATCAT
-1365    CCACATGTAAAAACCAGGATTAGTCAGCATAGCAGTGGTAAGGAACAAATAAGGAAGATAGATAT
-1300    AAGGAAAATAACAAACCATAATGGTGTTAAGATCCTCTTGAACAATATTTTCCAAAGAGACTTGT
-1235    TTAATGGCCACAAAGTCACCATTCTCCAAGTCTAATCCTTTATAAACTCGACCATAAGCTCCTTT
-1170    ACCAATTTCATCTCCCAGCATCTACCCTCAACATATCAAGGATCAACCATTGAAATTAATCAAAA
-1105    TTGAAGAGAAAACAGCAAAAAGCACCAGACTTAAGCTTAAAATCAATAAAATTACTAAAGCTAAA
-1040    ATCTTAAGGAGACCCATTAGAAAACCCAAATCCCAGAAATTACGGAGAAGAAGAAAGCTCACATA
-975     TTTGTTGTCGAGAGTCTTTGATTTGTGAAACTGAGATGACGTCATTTGCCGCGCCATCTCTTCTC
-910     GTTTCTTCTTCCTCCGTCAGCAGAACAAAAAAAATGCAGCGAACAGAACACAATTGCTCCTCAAG
-845     GAAACAGAATCCAATAAGAATTAGGGTTTTAATTTTTTGAGAAAATTGAAAGAAAAGATTTCGAG
-780     ATTGGTGGAGTTGAAAGATCAGAGATGCCAGCTCTATTACAGTAGTGAAAATACATGACCAAATT
-715     TTAATAAAATCAAAATTAAGAAAAAGGAAAGAACAAAACAAAAGTTTTGGGTTAGAAATTGACTA
-650     ACTGTAATAAAGGAGAGGTAATTATGTTAATTTCTCATGCGCCTGTATTCTTCTTTATTATGTGG
-585     CCCATACTTTTTATTTATTTACGATTTCACCCCTTTATTTCCCGATTTGTAAAAATACTAGCATT
-520     TTGTCCAATTACTGATTAATCATCTATTTAAAAGGTGGTCCAAAATATTAATGCTTTTTCACTGG
-455     TGGCAATTTTAATTAAGGTGGTTAGTATTCATTAATGTATTATCAACCATTTTGTTATAACTTTC
-390     TCAATTGTAGGAGCTGGTATAATCGGATGTTTATAGCCAATCAAATTATAATTCATAGTAGTATC
-325     AAATCTTTAATATAAATAACTAGAAGCAACTAACCTATGCATTGTAATAACATTAGCCATTTGGA
-260     TTTCAATATGATTGTTACTGCAAAATCTTTAAAATGAAAGGAAAAAAAAACCACGCAGGTGAAAT
-195     AAAAACCAAATGAAGATTATTACCAAACGCCGAAGCTAGCGCGTGAGAGAAAAGGTCGGTAGGTT
-130     CATTGGTAACATCAGCAGACTAATGGAGAGAGAGAAACTTTGAGAAAAGAAAAAAACAACCAATC
-65      GAAGAACTCATAGAGATCACGAAACCCTAGTGGAAAGAGAGAAAGCTTTGTTGAAGACGGCGGAG
+1       ATGATGTCATGTGGTGGGAAGAAGCCAGTGTCTAAGAAAACAACGCCGTGTTGCACGAAGATGGG
         M  M  S  C  G  G  K  K  P  V  S  K  K  T  T  P  C  C  T  K  M  G
+66      GATGAAGAGAGGACCATGGACGGTGGAGGAAGACGAGATTCTTGTGAGCTTCATTAAGAAAGAAG
          M  K  R  G  P  W  T  V  E  E  D  E  I  L  V  S  F  I  K  K  E  G
+131     GTGAAGGACGGTGGCGATCGCTTCCTAAGAGAGCTGGTTTACTCAGATGTGGAAAGAGCTGTCGT
           E  G  R  W  R  S  L  P  K  R  A  G  L  L  R  C  G  K  S  C  R
+196     CTACGGTGGATGAACTATCTCCGACCCTCGGTTAAACGTGGAGGAATTACGTCGGACGAGGAAGA
         L  R  W  M  N  Y  L  R  P  S  V  K  R  G  G  I  T  S  D  E  E  D
+261     TCTCATCCTCCGTCTTCACCGCCTCCTCGGCAACAGGTATATATATAGATATTAAAAAAAAATCT
          L  I  L  R  L  H  R  L  L  G  N  R
+326     CGTCAAGTATTCAACTATACAAGATCGTTTAGTTTATATGCATACGTATGTGTGTGTCTCTCTCT
+391     ACCCATACACTAGAGTTATTTTTTTGTTTAAGCTAGCTAGGGTTTTATTTCTTCCACATAAACAT
+456     GAAGGTTCTGATTTTAGTTTGATCCCATTTTTTCTTATAGCTATATAAAACAATTATACTTTGAT
+521     TCTTGATTCGATATTTATATAGAACAAAAGGTTAATCCCTTCTCTAGTTTCTTTAGAGTATAATT
+586     TTAATTAAGTTGATTCTGGTTCTAGAGCAAATTAATAGTTCTAGAGATCATTCATTCTTTTTTCA
+651     AATTGTTTATGATTACTAGGATAAAAATGCAAGCTTGTTTTGTAACCATATTAGTAATTAACCGC
+716     TCTTGTTCTTCATGGAAATTCTAATGTGTGATGCAGGTGGTCATTGATCGCGGGAAGGATACCGG
                                             W  S  L  I  A  G  R  I  P  G
+781     GAAGGACTGATAATGAAATTAAGAACTATTGGAACACTCATCTTCGTAAGAAACTTTTAAGGCAA
           R  T  D  N  E  I  K  N  Y  W  N  T  H  L  R  K  K  L  L  R  Q
+846     GGAATTGATCCTCAAACCCACAAGCCTCTTGATGCAAACAACATCCATAAACCAGAAGAAGAAGT
         G  I  D  P  Q  T  H  K  P  L  D  A  N  N  I  H  K  P  E  E  E  V
+911     TTCCGGTGGACAAAAGTACCCTCTAGAGCCTATTTCTAGTTCTCATACTGATGATACCACTGTTA
          S  G  G  Q  K  Y  P  L  E  P  I  S  S  S  H  T  D  D  T  T  V  N
+976     ATGGCGGGGATGGAGATAGCAAGAACAGTATCAATGTCTTTGGTGGTGAACACGGCTACGAAGAC
           G  G  D  G  D  S  K  N  S  I  N  V  F  G  G  E  H  G  Y  E  D
+1041    TTTGGTTTCTGCTACGACGACAAGTTCTCATCGTTTCTTAATTCGCTCATCAACGATGTTGGTGA
         F  G  F  C  Y  D  D  K  F  S  S  F  L  N  S  L  I  N  D  V  G  D
+1106    TCCTTTTGGTAATAtTATCCCAATATCTCAACCTTTGCAGATGGATGATTGTAAGGATGGGATTG
          P  F  G  N  I  I  P  I  S  Q  P  L  Q  M  D  D  C  K  D  G  I  V
+1171    TTGGAGCGTCGTCTTCTAGCTTAGGACATGACTAGATACAGGAAGATATATAGTTGATCGAATCT
           G  A  S  S  S  S  L  G  H  D
+1236    TGATGTCTTCTATATCTACCATGCTTCGAAGAAGGGTCACAGATGCGATCTAGAAGGTTGGCATA
+1301    TATGTGTACTAAGTTGTTTTGTGGTGATTTGTGTGATTATTATAAAACCATAAATTGTGAGTGTA
+1366    CTGAGTACACAAACTCTATTTATATATATGGATATATCTATTGCGAATAAATGAATCATCACTAT
+1431    CGTTAAATAAGACTCGTGGTAAGCTT
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Fig. 2. Nucleotide sequence of *Atmyb5*, the 5′ promoter region and the deduced amino acid sequence. The underlined amino acid sequences represent the conserved sequences also found in other plant Mybs. The underlined nucleotide sequences are putative signal sequences for polyadenylation. The single base difference in the allelic sequences of *Atmyb5* is represented by a small t at +1120.

```
                     Repeat R2

MKRGPWTVEEDEILVSFIKKEGEGRWRSLPKRAGLLRCGKSCRLRWMNYLRPS Atmyb5
Y-K-L------N--MDYVLNH-T-Q-NRIVRKT--K--------------S-N GL1
V---A--SK--DA-AAYV-AH---K--EV-QK---R----------L-----N C1
LIK----K---QRVIKLVQ-Y-PK--SVIA-HLK G-I--Q--E--H-H-N-E c-Myb

                     Repeat R3

VKRGGITSDEEDLILRLHRLLGNRWSLIAGRIPGRTDNEIKNYWNTHLRKK Atmyb5
-NK-NF-EQ-----I---K----------K-V------QV--------S-- GL1
IR--N-SY------I----------------L-------------ST-GRR C1
--KTSW-EE-DRI-YQA-KR-----AE--KLL------A---H--STM-R- c-Myb
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Fig. 3. Comparison of the Myb domain sequences. Atmyb5 sequence is compared with GL1 [2], C1 [3] and c-Myb [42]. The underlined sequence indicates the position of the 38-mer oligonucleotide used for library screening. The conserved tryptophan residues are highlighted with bold letters. Bars represent amino acids identical to those of Atmyb5.

*Atmyb5* mRNA as reprobing the blot with other genes gave sharp bands. The blot shown was exposed for 5 days and, taking into consideration the radioactivity of the probe and the amount of poly $A^+$ RNA loaded, this suggests *Atmyb5* mRNA is a low prevalence mRNA.

## 4. Discussion

A *myb*-related gene, *Atmyb5*, has been cloned from *Arabidopsis thaliana*. The gene contains a single intron in the region coding for the R3 repeat, disrupting the codon for arginine and having a location identical to many other plant *myb* genes such as barley *myb*Hv1, Hv5 and Hv33 [32]. The N-terminal sequence is typical of Myb sequences, consisting of two imperfect repeats, while the C-terminal sequence has no significant homology with known Mybs. However, two short sequences

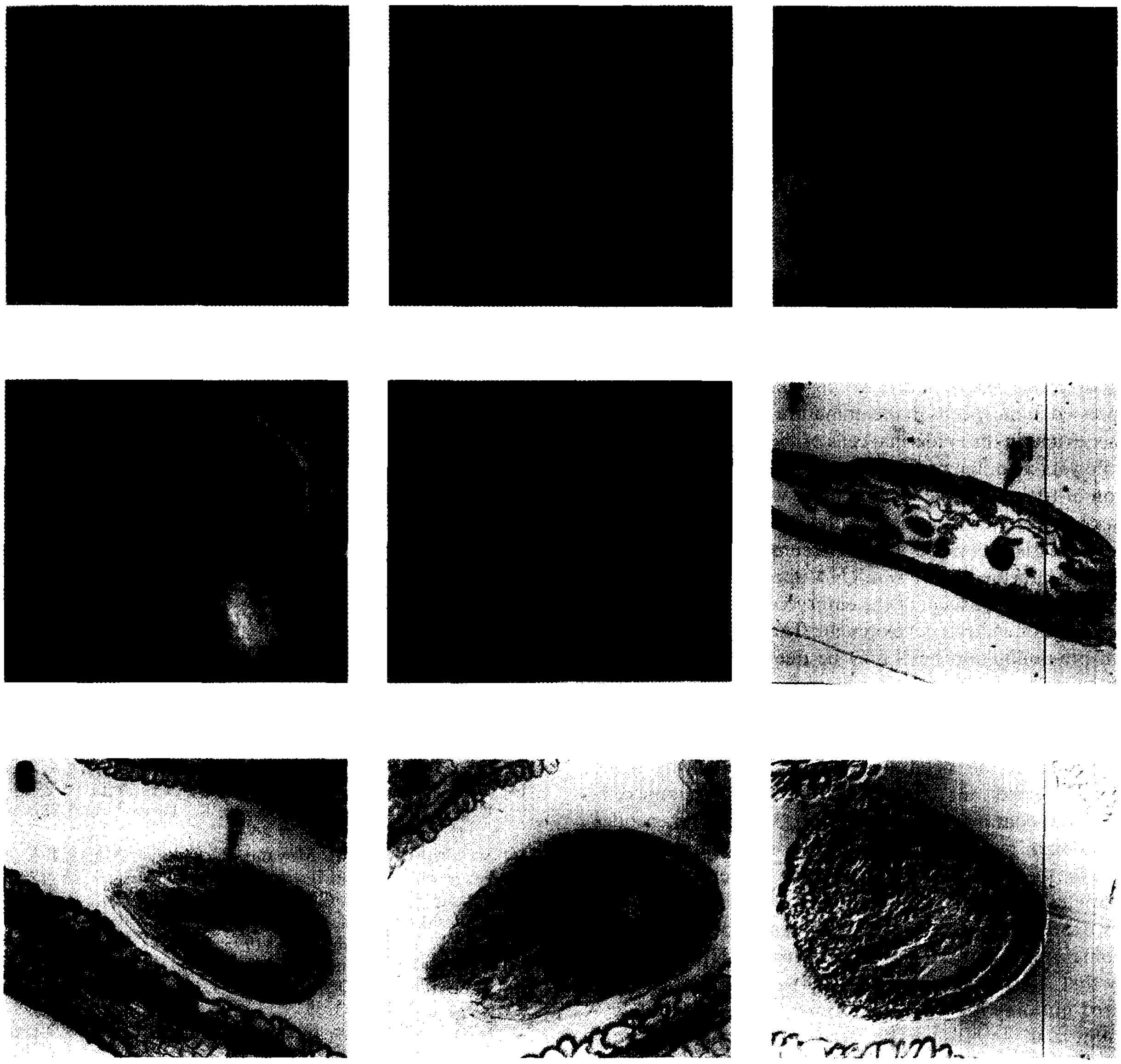

Fig. 4. Expression pattern of *Atmyb5-GUS* in tissues of transgenic *Arabidopsis*. Tissues were stained with X-gluc. (A) First pair of trichomes (tr) in first pair of leaves. (B) Young trichomes (tr) and stipules (st). (C) Seedling leaf margin (lm). (D) Trichomes in older leaves (weakly stained). (E) Young siliques (si). (F) Immature seeds (s). (G, H, I) Immature seeds (s) and epidermal cells of a silique.

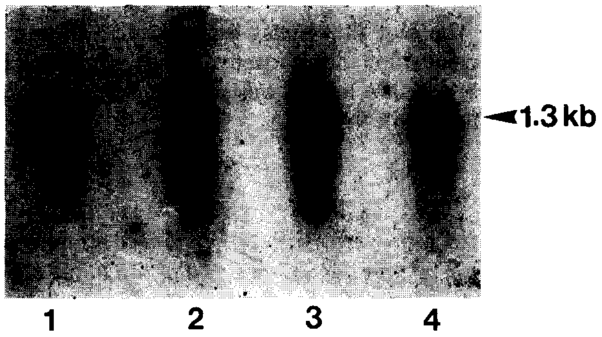

Fig. 5. Northern blot of *Atmyb5* mRNA at various stages of seed development. Concentrations of poly A$^+$ RNA used are shown in brackets. (1) Buds, including all floral stages to pre-pollination (0.5 μg). (2) Whole siliques, pistils at anthesis and collections of pistils until embryos at 16-cell stage (1.0 μg). (3) 16-cell stage to heart stage embryos (1.0 μg). (4) Heart stage to fully formed seed (1.0 μg).

within the C-terminal region are also conserved in some other plant Mybs. A short sequence of twenty amino acids at the N-terminus of Atmyb5 contains several serine and threonine residues which are potential phosphorylation sites. Phosphorylation of a serine residue within the short amino acid sequence at the N-terminus of c-Myb by a protein kinase inhibits the sequence-specific binding of c-Myb to DNA [33]. Dephosphorylation or deletion of the sequence restored the sequence-specific binding of c-Myb. Similarly, the DNA-binding activity of Atmyb5 might be regulated by a protein kinase that targets its N-terminal sequence.

Plants transgenic for *Atmyb5* promoter/*GUS* genes expressed the enzyme in developing trichomes of leaves, in stipules, on the margins of young leaves and in young siliques. GUS activity was expressed in most cells in the immature seeds observed and to a lesser extent in the epidermal cells of the siliques. Examination of flowers at different floral stages showed that, with the exception of trichomes, the GUS activity was not expressed in early stages before fertilization, i.e. the *Atmyb5* promoter was inactive. Analysis of *Atmyb5* mRNA accumulation during seed development indicated *Atmyb5* mRNA appears between fertilization and the 16 cell stage of the embryo and persists beyond the heart stage of embryo development. The very weak mRNA signal in pre-pollination buds may be due to the presence of trichomes on the sepals or expression in floral tissue below the levels detectable by the GUS assay.

Genes that influence aspects of both trichome and seed development have been identified in *A. thaliana*. Mutations in the *GL2* gene cause aberrant outgrowths of incipient trichome cells and effect seed coat mucilage production [34,35]. *GL2* encodes a homeodomain protein and is expressed in the surface layer of cells in the seed [36]. *GL1* (a *myb* homolog), is expressed only in the stipules and developing trichomes [37]. Unlike *Atmyb5*, it is not expressed in seeds and its sequence (other than the Myb domain) is quite different from *Atmyb5* [2]. Thus it is unlikely that *Atmyb5* is an allele of *GL1*. Another gene, *TTG*, is required not only for trichome formation but also anthocyanin synthesis in *Arabidopsis*, the production of seed coat mucilage and root epidermal cell fate and patterning [38,39]. Although the *TTG* gene has not been cloned, the *R* gene encoding a basic helix-loop-helix protein in maize compliments *Arabidopsis ttg* mutants [40] suggesting that *TTG* is, or regulates an *R*-homolog. Thus *Atmyb5* is unlikely to be *TTG* as Atmyb5 contains no basic helix-loop-helix motif. *Atmyb5* may cooperate with *TTG* in controlling trichome formation as occurs in the interaction between *C1* (*Myb*-like) and *R* required for maize anthocyanin synthesis [24]. Similarly, in combination with TTG (or an R homolog it regulates), Atmyb5 may regulate *GL2* in the seed testa [4]. GUS expression occurs in the inner and outer integument structures of the early seed which give rise to the testa.

*Atmyb5* expression occurs in the margins of young leaves and it will be of interest to determine if this is related to marginal meristem activity. It may also be relevant that trichomes on leaf margins do not appear to require *GL1* for development [35,41]. In situ hybridization and antisense experiments are currently being carried out to confirm the GUS data and study the function of *Atmyb5*. The role, if any, of the *Atmyb5* 3′ non-coding region in expression is also being examined.

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