A novel MYB-like gene (AtMYB103) was isolated from a genomic library of Arabidopsis. Plants transgenic for chimeric AtMYB103 promoter/GUS genes expressed the enzyme in early anthers. In situ hybridization of flower sections showed a high level of AtMYB103 mRNA in the tapetum and middle layer of developing anthers.

Key words: Anthers — Arabidopsis thaliana — MYB genes — Tapetum.

MYB-related proteins in plants are characterized by MYB domains consisting of one or two imperfect tandem repeats (R2 and R3). The MYB domains have been shown to bind to DNA in a sequence-specific manner both in animals (Ness 1996) and plants (Martin and Paz-Ares 1997, Romero et al. 1998, Urao et al. 1993, Sablowski et al. 1994, Solano et al. 1995, Li and Parish 1995, Goff et al. 1992, Cone et al. 1993). In Arabidopsis, the Glabrous-1 (GL1) gene is required for trichome formation (Larkin et al. 1993, Oppenheimer et al. 1991) and the Caprice (CPC) gene determines epidermal cell differentiation (Wada et al. 1997). Another MYB-like gene, the MIXTA gene from Antirrhinum is essential for the development of the conical form of petal epidermal cells (Noda et al. 1994). The cells of corolla lobes fail to differentiate into their normal conical form in mixta mutants. MYB proteins are also involved in hormonal responses. Expression of barley GAMYB is induced by gibberellic acid (Gubler et al. 1995). Abscisic acid induces the expression of AtMYB2 in Arabidopsis, a gene also induced by salt stress (Urao et al. 1993).

Relatively little is known about anther development at the molecular level. Many anther specific genes have been isolated (Rubinelli et al. 1998, Hamilton and Marscarenhas 1997) although little is known about the regulation of these genes by transcription factors. Isolation of genes encoding anther specific transcription factors will facilitate the understanding of anther and pollen development. In this paper, we describe a novel MYB-like gene from Arabidopsis thaliana and its expression in anthers.

A degenerate 38-mer oligonucleotide (5’-CCTGGTCG-TACTGA(C/T)AA(C/T)GA(A/G)ATTAA(A/G)AA(C/T)TA(C/T)TGAA-3’) covering the conserved region of the third repeats of the MYB DNA binding amino acid sequences (Fig. 1) was used to screen a genomic library of Arabidopsis thaliana (Landsberg erecta) in vector EMBL3 as described previously (Li and Parish 1995, Li et al. 1996). One of the positive clones identified (AtMYB103) was chosen for this study. To locate the region hybridizing to the 38-mer in the insert, the DNA was subjected to single and double digestion with restriction enzymes BamHI, EcoRI and Sall and probed with the 32P-labeled 38-mer in a Southern hybridization. A 1.5 kb BamHI-EcoRI fragment (AtMYB103) was identified and subcloned into the

The nucleotide sequence reported in this paper has been submitted to the GenBank under accession number AF048839.

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vector pTZ18. The region containing the 38-mer binding sites was sequenced using both the sequenase method (U.S. Biochemical Corp.) and the dye-primer method with a DNA sequencer. As the BamHI-EcoRI fragment contained only parts of the gene, a 2.5 kb EcoRI-EcoRI fragment and a 4.2 kb BamHI-BamHI fragment (AtMYB103) were subcloned and the remaining portions of the genes sequenced. To clone the cDNA copies, mRNA was isolated from total RNA of flower buds using oligo (dT)-Dyna-beads according to the procedure provided by the supplier (DYNAL). The mRNA bound to the oligo (dT)-Dyna-beads was transcribed using a reverse transcriptase. The mixture was subjected to PCR (polymerase-chain-reaction) with a pair of primers hybridizing to the sequences next to the start codon and to the stop codon, respectively. The corresponding PCR products were cloned and sequenced.

A 3.2 kb BamHI-SwaI promoter fragment of AtMYB103 was inserted into BamHI-Smal site of pBI101.1. The resultant fusion protein contains the first 46 amino acids from AtMYB103. To short the AtMYB103 promoter in the fusion construct, the fusion plasmid was digested with XbaI and HpaI, filled in and religated to obtain a 1.0 kb promoter fused to the GUS gene. The fusion plasmids were transformed into Agrobacterium tumefaciens strain AGL1 (Ditta et al. 1980, Armitage et al. 1988). Root explants of Arabidopsis thaliana (Landsberg erecta) were transformed with the Agrobacterium using the method described by Valvekens et al. (1988). T0 and T1 plants of six to twelve independent transformants from each transformation were obtained and subjected to detailed analysis. The incorporation of the AtMYB-GUS plasmid into the genomes was confirmed by PCR amplification of the promoter inserts from the genomic DNAs with a pair of primers hybridizing to the vector sequences (5'-TGTGGAATTGTGAGCGGATA-3', 5'-ATTCCACAG-)

The in situ hybridizations were carried out essentially as described by Drews et al. (1991) and Cox and Goldberg (1988). Early flowers were dissected and fixed in 50% ethanol, 5% acetic acid and 3.7% formaldehyde. The fixed tissues were dehydrated and embedded in paraffin (Sigma). The tissues were then sliced into 10 µm sections and attached to Superfrost plus slides (Menzel-Glaser, Germany). The sections were treated with xylene followed by hydration, proteinase K treatment, acetylation and dehydratation. The 35S-labelled probes were hydrolysed to about 100 nt in length and hybridized to the sections at 42°C for 17 h. The sections were then treated with ribonuclease A and washed, followed by emulsion of the slides.

The nucleotide sequence and the deduced amino acid sequence (Fig. 1) of the novel MYB gene were submitted to GenBank under the accession number AF048839. As this MYB gene is not included in the 102 MYB genes reported by Romero et al. (1998), it was named AtMYB103. The locations of the introns were confirmed by sequencing the cDNA copies of the genes. The gene contains two introns in the MYB region (Fig. 1). The N-terminal amino acid sequence is highly homologous to the MYB domains and

![Fig. 1](image-url)
Arabidopsis MYB and expression

Repeat R2

\[
\begin{align*}
\text{MGRIPCCEKENVKSKQWTPEDNKLASSIAQHGTKRNLIPKNAQQLQRGKCSRLWRTNLIRPD} & \quad \text{AtMYB103} \\
\text{---S----AHTN-A--K---ER-VA--RA--EGC--SL--A--L-------------I------} & \quad \text{ZmMYB38} \\
\text{---S--D-VGL-K-P-----Q--LA--EE--HGS--AL-AK---------------} & \quad \text{PhMYB1}
\end{align*}
\]

Repeat R3

\[
\begin{align*}
\text{LKHGQFSEAEHHIIVKFSVLGNRSWLSIAAQLPRTNDVKNYNTKLK} & \quad \text{AtMYB103} \\
\text{--R--N-TAD-DDL--L--L--K--R---E---T--------EV--------HVR--} & \quad \text{ZmMYB38} \\
\text{I--R--K-TLQ--QT-IQL--AL--A--TH--K--------} & \quad \text{PhMYB1}
\end{align*}
\]

Sequence adjacent to R3

\[
\begin{align*}
\text{LSGMGIDPVTHKPSH LMAEITTLNPPQVSHLAA} & \quad \text{AtMYB103} \\
\text{-L-R-----------R-IAA DAVTV--VSFQ-SP-AAA-A--} & \quad \text{ZmMYB38} \\
\text{-VR-------------KINDA-LSHDQSK-AANL--M-QWE} & \quad \text{PhMYB1}
\end{align*}
\]

Fig. 2 Comparison of the MYB domain sequence of AtMYB103 with ZmMYB38 of maize and PhMYB1 of petunia.

consists of two imperfect repeats, namely R2 and R3 (Fig. 2). The sequences at the carboxyl termini showed no significant homology with any of the other known MYBs. However, a short sequence within the carboxyl region (GIDPVTHKP) (Fig. 1) is identical or similar to the sequences found in AtMYB5, AtMYB6 and AtMYB7 (Li and Parish 1995, Li et al. 1996), in Antirrhinum MYB308, 315, 330 and MIXTA, barley MYB Hv1 and Hv33, and maize ZmMYB38, (Noda et al. 1994, Jackson et al. 1991, Marocco et al. 1989). The predicted molecular mass of AtMYB103 is 36 kDa with 320 amino acids. Southern analysis of restricted A. thaliana genomic DNA using the 3' region of the AtMYB103 revealed single bands with all restriction enzymes used (data not shown, R. Kalla, personal communication).

A BLAST search of the GenBank database revealed significant homologies between AtMYB103, ZmMYB38 and PhMYB1 both in the R2R3-MYB domains (65% identity) and the sequence immediately adjacent to the R3 domain (Fig. 2). The latter two genes are expressed in flowers. ZmMYB38 is involved in anthocyanin biosynthesis in maize (Marocco et al. 1989), while PhMYB1 is essential for the conical form of Petunia petal epidermal cells (Martin and Paz-Ares 1997).

To study the expression patterns of AtMYB103, a 3.2 kb or a 1.0 kb promoter fragment of AtMYB103 was fused in frame with the GUS gene in the binary vector. The two constructs were used to transform root explants of wild type Arabidopsis and several independent transformants from both constructs were examined for GUS expression. The incorporation of the AtMYB103-GUS constructs into genomes of these transformants was confirmed using PCR (data not shown). Plant tissues at various stages of growth were stained for GUS activity. The GUS expression patterns from both constructs were identical. GUS activity (blue color) was detected only in developing anthers of AtMYB103-GUS plants (Fig. 3A). No GUS activity was detected in the plants containing only the GUS vector (Fig. 3B). No GUS activity was detected in the very early flowers at the centre of the flower cluster (Fig. 3A, C1) which correspond to floral stage 8 (Bowman et al. 1993). The activity appeared in the young anthers of flowers at about floral stage 9 (Fig. 3C2, C3) in which pollen mother cells become separated from each other and from the tapetum. The pollen mother cells undergo meiosis to form tetrads of microspores. The GUS activity decreased as the flowers became more mature at floral stage 10 (Fig. 3C4, C5) and could not be detected at floral stages 11 and 12 (Fig. 3C6, C7, C8). Sections of the young anthers revealed that the GUS activity was expressed at a high level at the tetrad stage (Fig. 3D). The GUS activity was primarily expressed in the tapetum and the middle layer of anthers and to a lesser degree in microspores. Although claims have been made that the GUS assay might produce a false positive result in anthers (Block and Debrouwer 1992), we have not observed any GUS expression in anthers of plants expressing GUS activity from several other AtMYB promoter-GUS constructs. The in situ hybridization studies in flower sections revealed that AtMYB103 mRNA was expressed in the tapetum and the middle layer at high levels (Fig. 3E). A sense strand probe gave no detectable signal (Fig. 3F).
AtMYB103 expression commenced at about the onset of meiosis of the pollen mother cells. The tapetum is thought to provide nutrients, structural components and enzymes for microspore development. AtMYB103 may control some of the metabolic pathways involved in the synthesis of these compounds and proteins or regulate their secretion from the tapetum to the developing microspores. Since AtMYB103 is expressed during the early stages of anther development, it may also be involved in the formation, maintenance and breakdown of the tapetal and middle layers. Not surprisingly, AtMYB103 was also expressed in developing microspores. Several lipid biosynthetic genes are expressed both in tapetal cells and pollen grains (Piffanelli et al. 1997). AtMYB103 may regulate
metabolic pathways present in both tapetal cells and microspores and thus regulate pollen development. The importance of the tapetal cells in anther development is illustrated by the fact that many male sterile mutants are linked to tapetal dysfunction. Male-sterile plants have been obtained by expressing RNase in tapetal cells using tapetum-specific promoters (Mariani et al. 1990).

The effects on pollen development of inhibiting At-MYB103 expression using antisense and cosuppression methods are being examined.

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References


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