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Cloning and initial characterization of 14 *myb*-related cDNAs from tomato (*Lycopersicon esculentum* cv. Ailsa Craig)

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Abstract

myb-related transcription factors contain highly conserved DNA-binding domains. Using a mixture of degenerate oligonucleotides derived from the highly conserved region as probe, 14 *myb*-related clones were isolated from a cDNA library constructed using tomato hypocoyl mRNA. The expression of these clones was studied by northern blot analysis using poly(A)$^+$ RNA from 7 tissue types (hypocotyl, leaf, root, green and red fruit, immature and mature flower). This study has revealed a wide range of expression patterns which include multiple and single transcripts, some of which show marked tissue specificity. Two clones showing different expression patterns have been fully sequenced. The DNA-binding domains of these two tomato *myb* clones are compared with *myb* genes from other plant species and organisms. Of the three clones analysed so far by Southern hybridization, two are single-copy genes and one has multiple genomic copies.

Introduction

The first *myb* gene identified was that from the avian myeloblastosis virus (AMV) which causes acute monocytic leukaemia in chickens [39]. Subsequently, cellular homologues of the viral *myb* gene have been found in many organisms including mammals, insects, yeast and plants. Myb proteins bind DNA in a sequence-specific manner and can function as both transcriptional activators and repressors [4, 29, 41, 46]. In animals, *myb* genes have been shown to be involved in cell proliferation and differentiation [reviewed in 23, 25].

At least three functional domains have been defined in murine c-Myb protein. The DNA-binding domain is located at the amino-terminal end and consists of three imperfect repeats (R1, R2, R3) of 51 to 53 amino acids, with R2 and R3 being necessary and sufficient for both nuclear targeting and sequence-specific binding to DNA [4, 15, 16, 24, 30]. Three regularly spaced tryp-

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases.

tophan residues are conserved between the repeats, and have been suggested to play an important role in the function of the Myb proteins [1]. Recent NMR studies have shown that both R2 and R3 repeats contain three helices, the third helix in each being the recognition helix. When bound to the target DNA, the R2 and R3 repeats are closely packed in the major groove, such that the two recognition helices contact each other directly and thus bind the specific base sequences AACNG cooperatively [33]. The transactivation domain lies in the centre of c-Myb and is required for transcriptional activation [18, 41, 46], whereas the carboxyl-terminal negative regulatory domain has been found to down-regulate the c-Myb transactivation capacity [18, 41].

In plants, most *myb*-related cDNAs isolated so far from several different species have repeats R2 and R3 [2, 17, 22, 27, 34, 35]. However, one recently isolated *myb* gene from potato has only one repeat [3]. Such plant *myb*-related genes are believed to be involved in a diversity of functions including flavonoid biosynthesis [12, 35], trichome development [20, 34], dehydration stress [45] and cell shape determination [31]. For some of the plant Myb-related transcription factors the target genes have also been identified. For instance, the *C1* protein [35] activates transcription of anthocyanin biosynthetic genes in the aleurone and scutellum of maize kernel, this process also requiring the presence of a protein from the *R* gene family [6, 7, 38]. The *P* protein [11, 21] controls phlobaphone pigmentation in maize floral organs by activating the transcription of a subset of genes (*C2*, *CHI* and *A1*) involved in flavonoid biosynthesis [7, 12, 38], and the region of the *A1* promoter required for such P-activated transcription has been identified [12, 44]. A flower-specific Myb transcription factor from *Antirrhinum* (AM305) has also been shown to activate phenylpropanoid biosynthetic genes [40].

The aim of this work was to characterize *myb*-related genes in tomato as a prelude to understanding their function. Since *myb*-related genes have been shown to regulate anthocyanin biosynthesis, we used tomato hypocotyls as our staring material since anthocyanin pigmentation is readily observable in this tissue and several mutations have been identified which alter its intensity or distribution [36]. We report here the isolation and partial characterization of 14 *myb*-related cDNAs from tomato as a first step in elucidating their physiological functions in controlling plant gene expression.

Materials and methods

Plant materials

Seeds of *Lycopersicon esculentum* cv. Ailsa Craig were germinated under controlled environmental conditions (25 °C, 12 h light period). Three-week old seedlings were transferred to coir-based compost in individual pots and grown to maturity under the same conditions. Roots used for RNA isolation were grown in media containing MS salts [28], 30 g/l sucrose and 2% Phytagel (Sigma Chemical co., St. Louis, MO) in Magenta boxes at 25 °C under 12 h light period.

cDNA library construction and screening

Poly(A)$^+$ RNA was isolated directly from the crude extracts of hypocotyl tissue of 3-week old seedlings using Dynabeads oligo dT_{25} (Dynal, Norway). Double-stranded cDNA was synthesized using a cDNA synthesis kit and cloned into the *Eco*RI/*Xho*I sites of the UniZAPII vector according to the manufacturer's instructions (Stratagene, La Jolla, CA). The library was screened using a mixture of degenerate 38-mer oligonucleotides derived from the most highly conserved region of the *myb* R3 repeat sequences of the maize *C1* gene [35] and the six *Antirrhinum myb* genes [17]. The oligonucleotide sequences are 5′-T(TG)CC(CA)GGAAG(GA)AC(AC)-GA(TC)AATGAAATCAAGAA(TC)TA(CT)-TGG-3′. The oligonucleotides were end-labelled with γ-^{32}P-ATP and hybridization carried out in 0.5 M sodium phosphate buffer (Ph 6.7–7.0) containing 7% SDS and 5 mM EDTA at 55 °C for 12 to 16 h. The filters were washed in 2 × SSPE, 1% SDS at 55 °C for 30 min. The inserts

of hybridizing clones, together with the vector sequence of phagemid pBluescript SK^-, were isolated following *in vivo* excision, yielding circular phagemids, according to manufacturer's instructions (Stratagene, La Jolla, CA).

Isolation of RNA and DNA

For northern blot analysis, total cellular RNA was isolated according to the method of Hall *et al.* [13] and poly(A)$^+$ RNA isolated from total RNA using Dynabeads oligo dT_{25}. Total genomic DNA was isolated from tomato leaves using a modification of the CTAB method described by Doyle and Doyle [9].

Northern and Southern blot analysis

Electrophoresis and transfer of poly A^+ RNA was performed according to Davis *et al.* [8]. The RNA was cross-linked to Hybond N^+ membrane (Amersham, UK) by UV irradiation using the 'auto-cross-link' setting on a Stratalinker (Stratagene, La Jolla, CA). Genomic DNA was digested with appropriate restriction enzymes overnight at 37 °C and electrophoresed in a 0.8% agarose gel in 0.5 × TBE buffer. The treatment and transfer of the DNA under alkaline conditions were performed as described by Brown [39]. Probes used for northern analysis consisted of the complete cDNA insert, purified from the pBluescript vector sequence following restriction with enzymes which cleave within the polylinker but not within the cDNA sequence. For Southern analysis, the probes consisted of the gene-specific region 3′ of the *myb* repeats. Hybridization was carried out overnight at 60 °C for northerns and 65 °C for Southerns in buffer containing 0.5 M sodium phosphate buffer (pH 6.7–7.0), 7% SDS and 5 mM EDTA. The filters were washed in 2 × SSPE, 1% SDS at the same temperature as the hybridization for 30 min initially and where necessary, a further wash was carried out at this temperature for 10 min either in 0.5 × SSPE, 1% SDS or in 0.1 × SSPE, 1% SDS.

Sequencing

Nested sets of 3′ and 5′ deletions of the cloned insert were constructed using *Bal*31 nuclease. Double-stranded DNA sequencing was carried out on both strands according to the manufacturer's instructions using Sequenase Version 2.0 (United States Biochemical Corporation, Cleveland, OH) and computer analysis of DNA sequences performed with the PC/GENE DNA and protein analysis package (Intelligenetics, Mountain View, CA).

PCR amplification and analysis of products

PCR reactions were carried out in an Perkin Elmer Cetus DNA Thermal Cycler. Genomic DNA (100 ng) isolated from tomato leaf tissue was used as template for 30 cycles of PCR amplification (94 °C, 1 min; 52 °C, 30 s; 72 °C, 30 s) using primer sequences 5′-ACTCAAGAGCTTCGTCTT-3′ and 5′-GTATAGTTTCTTAAATACTCT-3′. The reaction mixtures contained 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 200 μM each dNTP, 0.4 μg each oligonucleotide primer and 0.25 U *Taq* polymerase. The DNA fragment amplified was sequenced directly as described above.

Results

Construction of hypocotyl cDNA library and isolation of myb*-related clones*

A cDNA library made from hypocotyl mRNA was cloned into the UniZAPII vector giving a titre of 2.9×10^6 plaque-forming units (pfu)/ml. About 6×10^5 pfu from unaplified library were screened using a mixture of degenerate oligonucleotides derived from the sequences of the maize *C1* and six *Antirrhinum myb* genes at the highly conserved Myb DNA-binding domain. Twenty-one positive clones were identified and purified.

Partial sequencing of the inserts confirmed that all clones were poly-adenylated and showed the

presence of the conserved *myb* imperfect repeats close to the 5′ end, thereby confirming that all clones are *myb*-related (data not shown). Comparison of sequences immediately adjacent to the *myb* repeats indicated that six clones were duplicated. Therefore, 14 of the *myb*-related clones isolated from the tomato hypocotyl cDNA library are independent and were named THM clones (short for tomato hypocotyl Myb). By comparison to the N-termini of the *Antirrhinum myb* genes [17], six of these THM clones were judged to carry a full length coding region, the others being partial clones containing varying length 5′ deletions of repeats R2 or R3.

Expression of the 14 tomato myb*-related genes*

The expression of the 14 independent clones was investigated in 7 different tissues types (hypocotyl, leaf, root, green and red fruit, immature and mature flower) by northern blot analysis using the complete cDNA sequence as probes. The results (Fig. 1) indicated that the expression of *myb*-related genes from tomato fell into several broad groups. The first group (THM1, THM6, THM9, THM10 and THM16) showed strong hybridization to multiple transcripts in most tissues investigated, the expression level of these transcripts varying between the different tissue types. Some of the transcripts appeared to be tissue specific or predominantly expressed in one tissue. For example, probes derived from THM1, THM6, THM9 and THM16 all hybridized to a ca. 0.9 kb transcript abundant in green fruit, but which was very low or absent in the other tissues tested. Interestingly, the pattern of hybridization produced with probes from THM1, THM6 and THM16 was very similar, despite the sequences of these clones being quite distinct (data not shown).

The second group (THM18, THM23 and THM24) showed hybridization to two transcripts, being of highest abundance in root tissue when probed with THM18 or THM24, whereas the THM23 probe hybridized most strongly to transcripts in leaf tissue. Al three clones were isolated from a hypocotyl cDNA library, and thus would be expected to hybridize to at least one transcript from this tissue. However, no signal was detected with THM23 or THM24 probes under the experimental conditions used. It may therefore follow that, in other tissues such as fruit or flowers, these genes could also be expressed at a low but undetected levels.

The third group (THM3, THM5, THM13, THM25, THM26 and THM27) showed hybridization to a single transcript. Probes from THM25 and THM27 hybridize to a transcript present in the majority of tissue types whereas, at the other extreme, a probe from THM5 appears to hybridize to a unique transcript seen only in root tissue. However, as discussed above, low level expression may exist in other tissues, since the expected expression in hypocotyl tissue was not detected for probes derived from THM3 or THM5 under the conditions used.

Full sequence of two tomato myb *cDNA clones*

The complete nucleotide and deduced amino acid sequences of two full-length clones showing different expression patterns (THM1 and THM27) are shown in Fig. 2. Clone THM1 is 1352 bp in length and the predicted ORF of 993 bp encodes a polypeptide of 331 residues and molecular weight of ca. 38 kDa. However, clone THM27 was 1132 bp in length but had an unexpectedly short ORF of only 96 bp. Comparison of the THM27 cDNA sequence with the other clones isolated from tomato indicated a possible frameshift and resultant stop codon after the 32nd amino acid resulting in severely truncated putative polypeptide product. In order to determine whether this stop codon was also present in the genomic copy of THM27, the corresponding region was amplified by PCR using tomato genomic DNA as template (see Materials and methods). Direct sequencing of the resultant PCR product clearly indicated that the genomic copy contained two extra nucleotides (TG; indicated in lower case in Fig. 2) at position 206–207 restoring the reading frame to that expected. Thus, the ORF of the

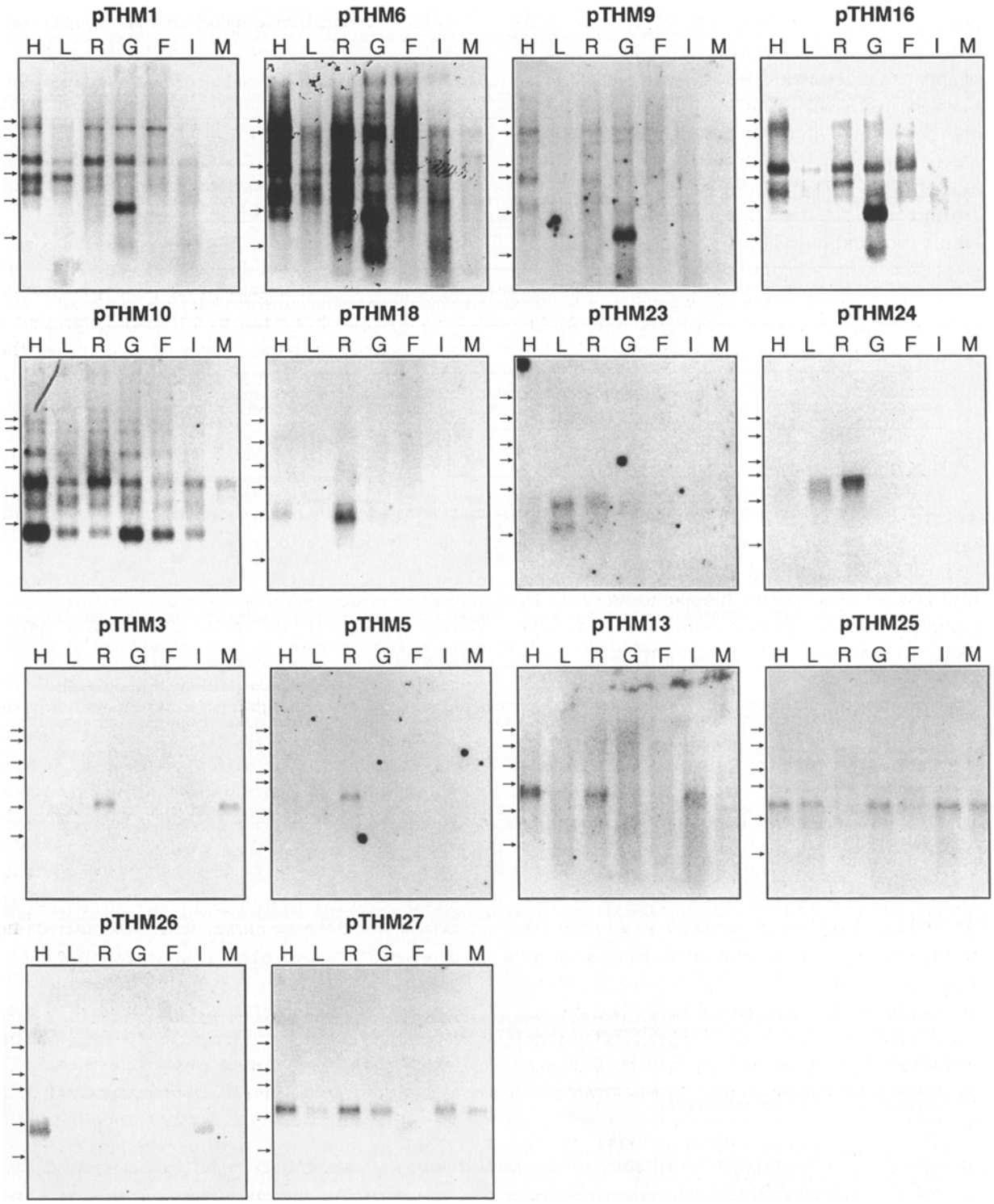

Fig. 1. Expression of the 14 tomato *myb*-related genes elucidated by northern blot analysis. A 1 μg portion of poly(A)$^+$ RNA isolated from hypocotyl (H), leaf (L), root (R), green fruit (G), red fruit (F), immature flower (I) and mature flower (M) tissue was loaded in each lane. Molecular weight markers indicated by arrows are 0.52, 1.0, 1.6, 2.0, 3.1 and 4.1 kb single-stranded DNA.

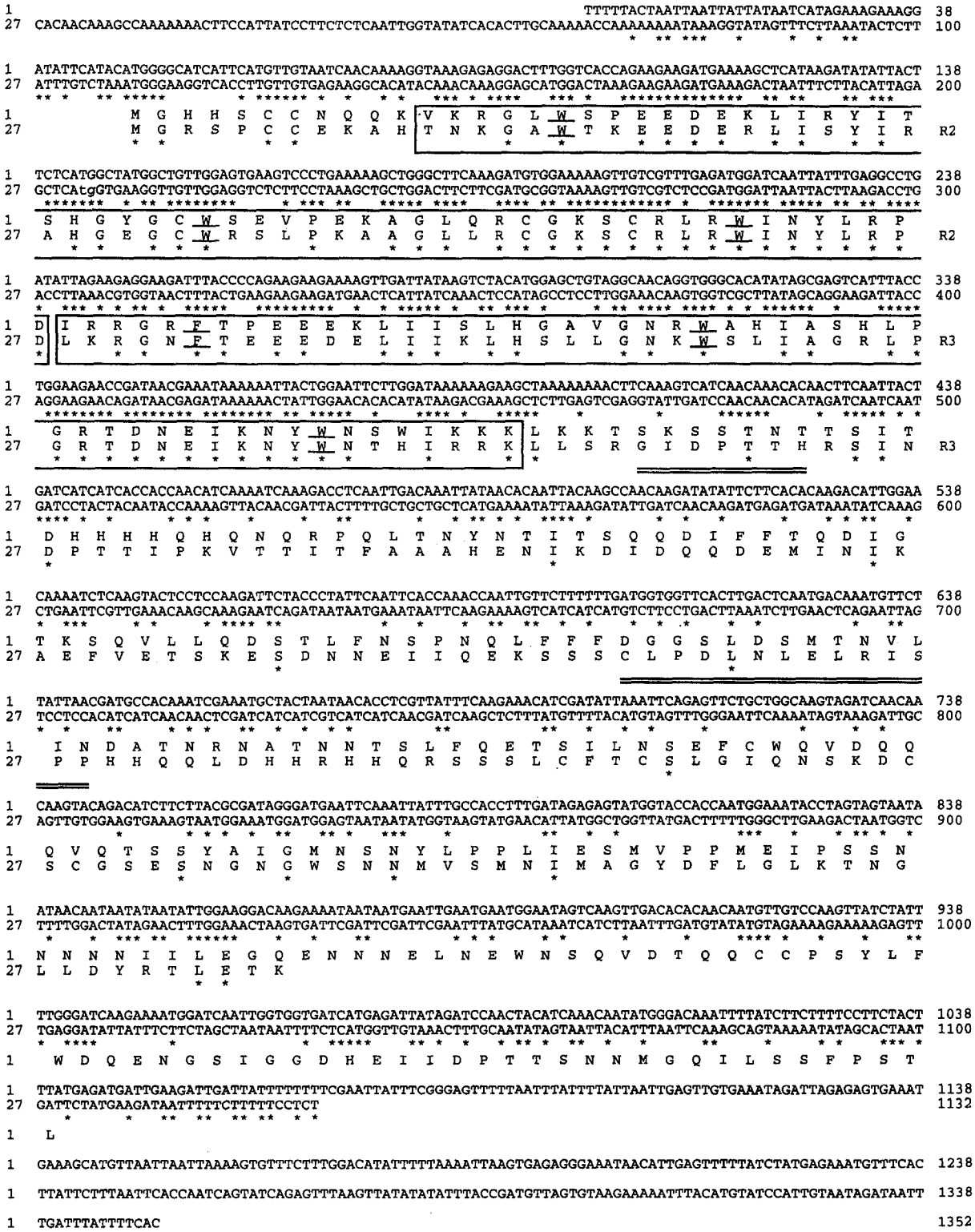

Fig. 2. Complete nucleotide and deduced amino acid sequences of THM1 and THM27. DNA sequences are aligned from the assumed initiation codon and given with the deduced amino acid sequences below. Conserved nucleotide or amino acid residues are indicated by an asterisk below. The conserved R2 and R3 DNA-binding repeats are boxed, the repeated tryptophan/phenylalanine residues within these repeats being underlined. Lower-case letters indicate the two nucleotides missing from the isolated cDNA clone THM27, but present in the genomic gene (see Results). Motifs in THM27 are double-underlined (see Discussion).

genomic copy of THM27 is predicted to be of 819 bp and encodes a polypeptide of 273 amino acid residues and molecular mass of ca. 31 kDa.

The characteristic Myb repeats (51 to 53 amino acid imperfect repeats; boxed in Fig. 2) and the regularly spaced tryptophan residues, which have been implicated in the binding of DNA [1] are evident in the protein sequences of both clones. The first tryptophan residue of repeat R3 in these two tomato *myb*-related genes is substituted by phenylalanine residue. This is in keeping with other plant *myb*-related genes, where the first tryptophan of this repeat is often replaced by another hydrophobic residue (isoleucine, leucine or phenylalanine).

Since the most conserved region in Myb proteins is the DNA-binding domain, these regions from THM1 and THM27 were compared with the same region of the Myb proteins from other plant species and organisms. The degree of similarity among these sequences (Fig. 3) indicates that THM1 and THM27 are more similar to Myb-related proteins from other plant species than they are to one another. The DNA-binding domains of THM1 and THM27 exhibit only 64% similarity, whereas those regions of THM27 and AM308 from *antirrhinum* [17] are 96% similar, and the value for THM1 and Hv33 from barley [47] is 72%. Outside the DNA-binding domain, no extensive amino acid similarity is evident between the proteins encoded by THM1 and THM27. The polypeptide encoded by THM27 shows similarity to that encoded by AM308 and the barley Hv1 gene [27] at several positions. Firstly, at the amino terminal region of all three proteins, a region of 22 amino acids in length (11 of which lie within the R2 box) is identical. Furthermore, two conserved regions downstream of

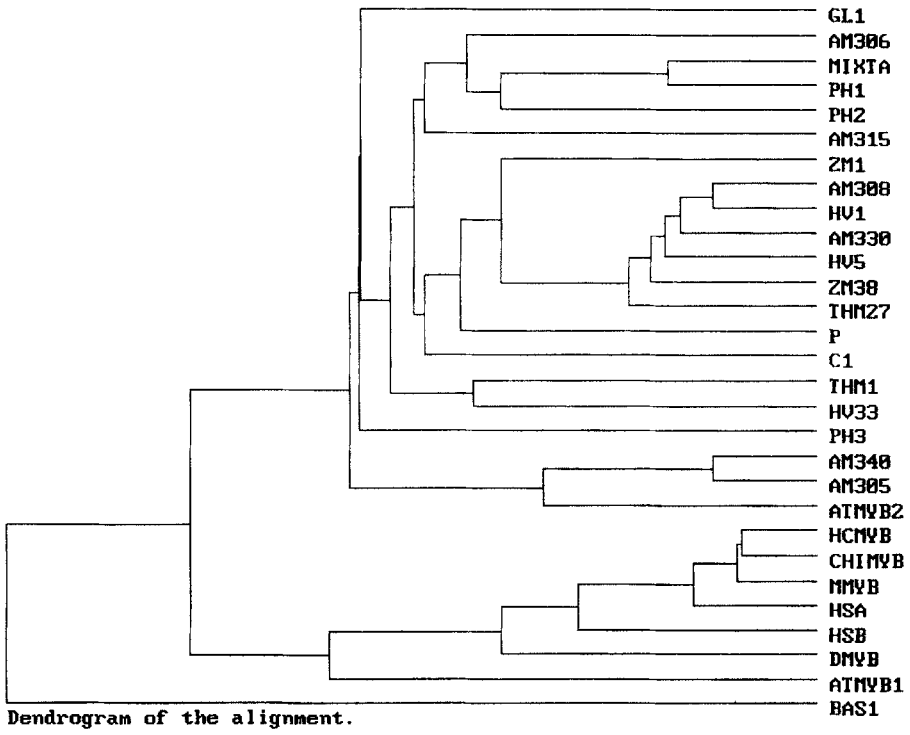

Fig. 3. Amino acid sequence comparison of R2 and R3 repeats of THM1 and THM27 with those of other Myb proteins. Dendrogram was calculated using the CLUSTAL method [14]. Sequences of the Myb DNA-binding domains are from: *Arabidopsis*: GL1 [34], ATMYB1 [42], ATMYB2 [45]; *Antirrhinum*: AM305, AM306, AM308, AM315, AM330 and AM340 [17], *mixta* [31]; maize: C1 [35], P [11], Zm1, Zm38 [27]; barley: Hv1, Hv5, Hv33 [27]; petunia: PH1, PH2 and PH3 [2]; human: HCMYB [26], HSA, HSB [32]; mouse: MMYB [10]; *Drosophila*: DMYB [19]; chicken: CHIMYB [37]; yeast: BAS1 [43].

the Myb repeats have also been identified in all three proteins. The first sequence, GIDPTTH in both THM27 and AM308, and GIDPVTH in Hv1, is similar to that found in other Myb proteins (GIDPXXH) from *Antirrhinum* (AM315 and AM330), barley (Hv33), maize (Zm38 [27]) and petunia (MYB.Ph1 [2]). The second region, CLPDLNLELRISPP (amino acids 186–199) in THM27 is similar to CPDLNLDLKISPP in AM308 and CPDLNLDLCISPP in Hv1. A related sequence (CPDLNLDLXISPP) is also found in Myb protein Zm38.

Southern blot analysis

The genomic organisation of THM1, THM18 and THM27 (a representative from each group showing different expression patterns) was studied by Southern blot analysis using the unique 3′ region (excluding the R2/R3 repeats) of the corresponding cDNA as probes. As shown in Fig. 4, the gene-specific probes of THM1 and THM18 detected a single copy of the gene, whereas the specific probe of THM27 detected multiple copies of related sequences. As expected, two bands were detected by the specific probes of THM1 and THM18 when *Sca*I was used to digest the genomic DNA since *Sca*I cuts within the genomic region covered by the probes used.

Discussion

We have isolated 14 different *myb*-related cDNAs from tomato, which represents the largest number so far isolated from one species. It is believed that plant species contain large numbers of *myb*-related genes; the genome of *Arabidopsis* has been estimated to contain at least 12 *myb*-related genes [45] and six different *myb*-related cDNA clones have been isolated from *Antirrhinum* flower tissue alone [17]. All the clones isolated from tomato contained repeats R2 and R3 characteristic of the DNA-binding region of the other *myb* genes [41, 46]. However, it is evident both from sequence outside this conserved region and the expression patterns, that the clones are diverse both in structure and tissue specificity, and therefore presumably also exhibit a diversity of function.

Northern analysis of the 14 tomato *myb*-related clones indicated a wide range of both transcript size and expression level in a variety of tissues. This diversity of expression pattern, and the lack of any hybridizing transcripts common to all clones, indicates that although the probe sequences contain the conserved R2/R3 repeat regions (THM1 and THM27 share 64% similarity at DNA level), these do not cross-hybridize under the conditions used. Furthermore, where investigated, the expression patterns were the same when either the complete cDNA sequence or a 3′-specific region was used as probe (data not shown).

Although none of the *myb*-related clones isolated was expressed in only one tissue, there is evidence of tissue-specific expression of some of the transcripts, for instance the prominent transcript in green fruit for THM1, THM6, THM9 and THM16. Furthermore, several of the clones, such as THM3, THM5, THM18 or THM24, express only one or two transcripts in a very limited number of tissues, whereas, other clones (e.g. THM25 and THM27) are expressed more uniformly in the majority of tissues tested. It is worth noting, however, that expression levels of *myb*-related genes may be relatively low and thus it is possible that, under the conditions used, expression in some tissues may remain undetected. This is likely to be the case for the expression of THM3, THM5, THM23 and THM24 in hypocotyl tissue, since although all the clones were isolated from a hypocotyl cDNA library, these clones do not appear to hybridize to any transcripts from this tissue.

Clones with contrasting expression patterns were chosen for further analysis: THM1 is representative of the group of clones which hybridize to multiple transcripts, and THM18 and THM27 which hybridize to two and one transcript, respectively. Southern analysis indicated that both THM1 and THM18 are single-copy genes (Fig. 4). In both cases *Sca*I cuts within the region covered by the probe thus giving two hybridizing

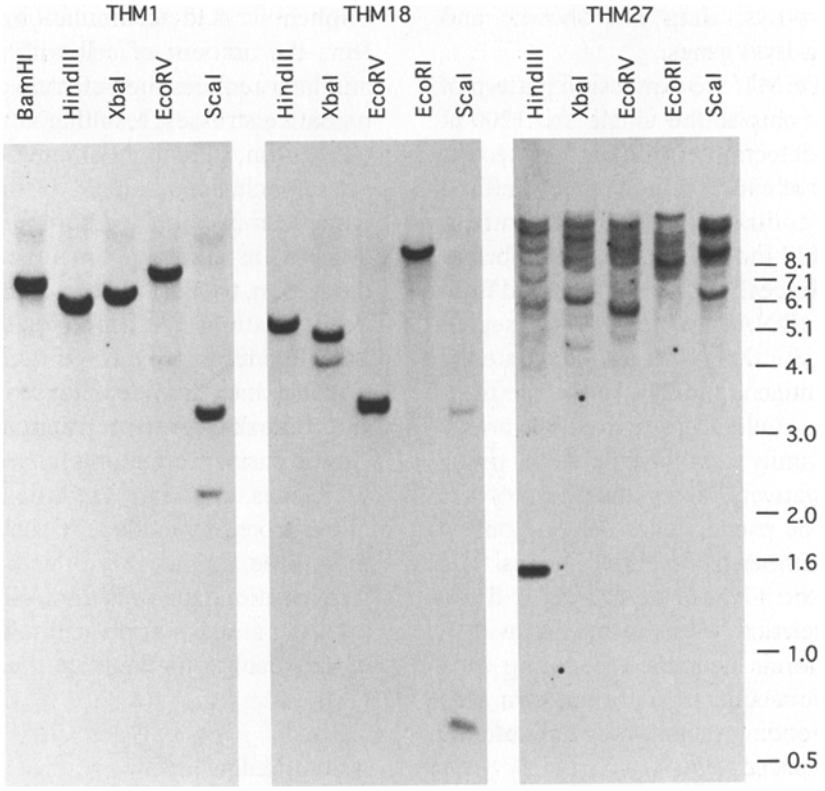

Fig. 4. Southern blot analysis of THM1, THM18 and THM27. About 10 μg genomic DNA was digested with appropriate restriction enzymes as indicated for each lane. The molecular weight of DNA markers are indicated in kb.

fragments. It is interesting that, in the case of THM1, a single genomic copy gives such complex expression pattern. At least seven different length transcripts are produced, the relative expression of which varies between the tissues studied; one transcript of ca. 900 nt is highly and specifically expressed in green fruit, whereas another transcript of about 2000 nt is expressed in all tissue types tested. These multiple transcripts could result from alternative splicing, incomplete processing of RNA, utilization of different promoter sites or premature termination depending on the signals present in the different tissues. It would appear that such factors influence the expression of THM1, THM6, THM9 and THM16 in a similar fashion, although it is clear from the sequence data available (data not shown) that these clones are different at the DNA level, are thus likely to be derived from independent genes. The existence of the multiple transcripts and their differential expression in different tissues indicates that the regulation of the expression of these *myb* genes is likely to be complex. Indeed, multiple transcripts have been observed for the *P* gene. The largest transcript being some 7000 nt in length was suggested to be an unprocessed or incompletely processed RNA and the other two transcripts were shown to be alternatively spliced at the 3′ end of the gene [11].

The complete nucleotide sequence of THM1 revealed that the putative protein contains the characteristic R2 and R3 Myb-repeats with evenly spaced tryptophan residues, as well as the basic cluster of amino acid (Lys-Lys-Lys) observed at the C-terminus of the R3 repeat of other Myb proteins. This basic cluster occurs within the five amino acids at the C-terminus of the mouse c-Myb R3 repeat, deletion of which abolishes recognition of the Myb-responsive element [15] and also is observed in the proteins encoded by

THM18 (Lys-Lys-Lys; data not shown) and THM27 (Arg-Arg-Lys) genes.

In contrast to THM1, the expression pattern of THM27 appears simple, the single ca. 1200 nt transcript being detectable in all tissues tested, although the level of expression in leaves and red fruit is low. In contrast to THM1, Southern analysis of THM27 indicates a large number of hybridizing loci. Since the probe was derived from the 3′ end of the cDNA and lacks the conserved R2/R3 repeat region, this result may indicate the existence of a multigene family. The single transcript in all tissues could indicate that the various genes within the family each have a narrow tissue specificity. Alternatively, some members of the gene family may be pseudogenes, leaving only a relatively small number of functional genes. The observation that the THM27 cDNA clone has a two nucleotide deletion when compared with a PCR-derived genomic sequence, resulting in a frame-shift and consequent generation of a stop codon within the coding region, may indicate the presence of such pseudogenes.

Several sequence motifs are shared by the deduced protein encoded by THM27 and other plant Myb proteins, possibly indicating a structural basis for functional similarity. The dendrogram based on amino acid similarity within the DNA-binding repeats (Fig. 3) indicates that this region of THM27 is most similar to those of two barley (Hv1 and Hv5 [47]) and two *Antirrhinum* (AM308 and AM330 [17]) Myb proteins. Interestingly, outside this conserved region THM27 has two more motifs, GIDPTTH and CLPDLNLELRISPP, which are also present in the same four Myb proteins (with the exception of the latter motif in AM330). In transgenic tobacco, chimeric Myb-GUS fusions with Hv1 and Hv5 promoters are both expressed in many tissue types. The GUS activity of extracts are fairly uniform for the various tissues tested; the exceptions are low expression in leaf tissue and very high expression in roots and at leaf nodes [47]. This pattern appears somewhat reminiscent of the expression pattern observed for THM27. When the *Antirrhinum* AM308 gene is overexpressed in transgenic tobacco, the phenotype appears to be the inhibition of phenolic acid accumulation, which in turn, affects the amount of cell wall material in leaves and later reduces the buffering capacity of cells to oxidative stresses, resulting in necrotic cell death (C. Martin, personal communication).

In conclusion, plant Myb transcription factors have been found to be both numerous and to be involved in a diversity of functions such as pigmentation, trichrome development and cell shape determination. We have isolated a collection of 14 different tomato *myb*-related cDNA sequences which exhibit a wide diversity of tissue expression. The data presented here indicate that all the clones carry the features characteristic of other *myb* genes, but so far say little about the function of the proteins encoded. We believe it highly likely that these tomato Myb-related proteins act as transcription factors in controlling target gene expression and are at present taking a wide variety of approaches to elucidate their functions.

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