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Molecular analysis of *tap2*, an anther-specific gene from *Antirrhinum majus*

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Deficiens is a floral homeotic gene of *Antirrhinum majus*, mutation of which results in transformation of petals to sepals and stamens to carpels. In a search for putative target genes, controlled by this regulatory locus, cDNA clones representing genes, that are expressed in wild type but not in the *deficiens* mutant flowers, were isolated by differential screening. The molecular structure and the expression pattern of one of these genes, *tap2*, is described. *Tap2* is transiently and tissue-specifically expressed in the tapetum of the anthers. It encodes a 131 amino-acids-long protein with a hydrophobic N-terminus, displaying all characteristic features of a signal peptide. This indicates that the *TAP2* protein may be secreted.

Flower development; *Antirrhinum majus*; Signal peptide; Tapetum; Hybridization, in situ; *Deficiens*

1. INTRODUCTION

Deficiens is a homeotic gene involved in the genetic control of flower development in *Antirrhinum majus*. Upon mutation of this regulatory gene transformation of petals into sepals and stamens into carpels occurs [1]. *Deficiens* encodes a protein that shows strong homology to the DNA-binding domain of the transcription factors SRF from mammals and MCM1 from yeast. This suggests that *deficiens* regulates the expression of genes necessary for petal and stamen formation [2]. In an attempt to isolate putative target genes of *deficiens* a cDNA library enriched for young inflorescence-specific cDNA clones was differentially screened with (+)probes prepared from mRNA of young wild type inflorescences and with (−)probes made from mRNA of young inflorescences of the *deficiens* ^{globifera} (*def* ^{gli}) mutant. Sixty-eight cDNA clones were isolated that hybridized specifically with the (+)probe, but not with the (−)probe [2]. Based on crosshybridization the cDNA clones were assigned to 12 distinct groups (1–12) representing 12 different genes that were characterized further.

In this report we present the DNA sequence of one of the genes *tap2*, and the deduced amino acid sequence of the *TAP2* protein. In addition, the temporal and spatial expression pattern of *tap2* is reported and its possible regulation by the floral homeotic gene *deficiens* is discussed.

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The nucleotide sequence data presented in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence databases under the accession number X55434.

2. MATERIALS AND METHODS

2.1. *Plant material*

Plants were grown in the glasshouse at 20–25°C under additional light during winter. The *deficiens* mutants are described by Klemm [1] and by Sommer et al. [2]. As wild type the *A. majus* line Sippe 50 was used for all cloning experiments.

2.2. *Isolation of plant DNA and RNA*

Genomic DNA from plants was isolated following the procedure described by Schwarz-Sommer et al. [3]. Total RNA was prepared from frozen tissues according to Logemann et al. [4]. Subsequently poly(A)$^+$ mRNA was isolated by chromatography on oligo-dT cellulose, following the protocol of Sommer et al. [5].

2.3. *Blotting techniques*

After agarose gel electrophoresis RNA and DNA were transferred to Hybond N filters (Amersham) by standard techniques [6]. Hybridization with radioactive probes was carried out overnight at 42°C in 50% formamide and 5 × SSPE for Northern blots, and at 68°C and 5 × SSPE for Southern blots. DNA probes were labelled by 'random priming' according to the protocol supplied by Amersham.

2.4. *Genomic cloning and cDNA cloning*

A genomic library was constructed in EMBL4 phages [7] following the protocol described by Sambrook et al. [6]. CsCl-purified genomic DNA was partially digested with *Mbo*I, size fractionated by gel electrophoresis in 0.6% agarose and fragments of 17–23 kb in size were ligated with the EMBL4 arms. The ligation mix was packaged in vitro, 200 000 recombinant phages were plated out and screened with probes prepared from the cDNA clones.

Cloning of the cDNAs was described by Sommer et al. [2].

2.5. *Sequence analysis*

The isolated phages were mapped by restriction analysis and fragments representing *tap2* subcloned into pUC18 or pBR322. DNA fragments were dephosphorylated with bacterial alkaline phosphatase and end-labelled using polynucleotide kinase and [γ-^{32}P]ATP. Subsequently, sequencing reactions were performed using the chemical method of Maxam and Gilbert [8].

2.6. *In situ hybridization*

The *tap2* cDNA was subcloned in Bluescript plasmid vector (Stratagene). To obtain ^{35}S-labelled antisense RNA this plasmid was linearized and used as a template for transcription with the appropriate (T7 or T3) RNA polymerase in the presence of [^{35}S]UTP.

Inflorescences and small flower buds were embedded in paraffin after fixation in ethanol/acetic acid. Longitudinal sections of inflorescences and cross sections of flower buds (8–10 μm thick) were mounted on gelatin coated slides. Pre-treatment of slides, hybridization, post-hybridization washes and autoradiography were performed according to Huijser et al. (in preparation).

After exposure slides were developed, stained with Fluorescent Brightener (C.I. 40622) and covered with Entellan (Merck). Distribution of silver grains was studied microscopically using dark field optics. Underlying tissue was visualized by epifluorescence.

3. RESULTS AND DISCUSSION

Differential screening of a subtracted cDNA library prepared from young wild type inflorescences (3–8 mm in length), using (+)probes from wild type and (−)probes from mutant inflorescences, yielded 12 distinct groups of cDNAs [2]. Group II, representing the *tap2* gene (see below), comprised 9 crosshybridizing members. The insert (about 300 bp in length) of one of these members was used as radioactive probe for isolation of nearly full-size cDNA clones and of genomic clones from conventional cDNA and genomic libraries.

In order to determine the structure of the *tap2* gene, several genomic and cDNA clones were analysed by DNA sequence analysis. The longest *tap2* cDNA insert is 510 bp in length, including a 30-bp poly(A) tail. The first ATG is embedded in a context (TATCATGGC) that agrees with the consensus sequence of plant translational start sites in the important positions [9,10]. The deduced *TAP2* protein is 131 amino acids long and has a hydrophobic N-terminus which displays all the characteristics of a signalpeptide [11], indicating

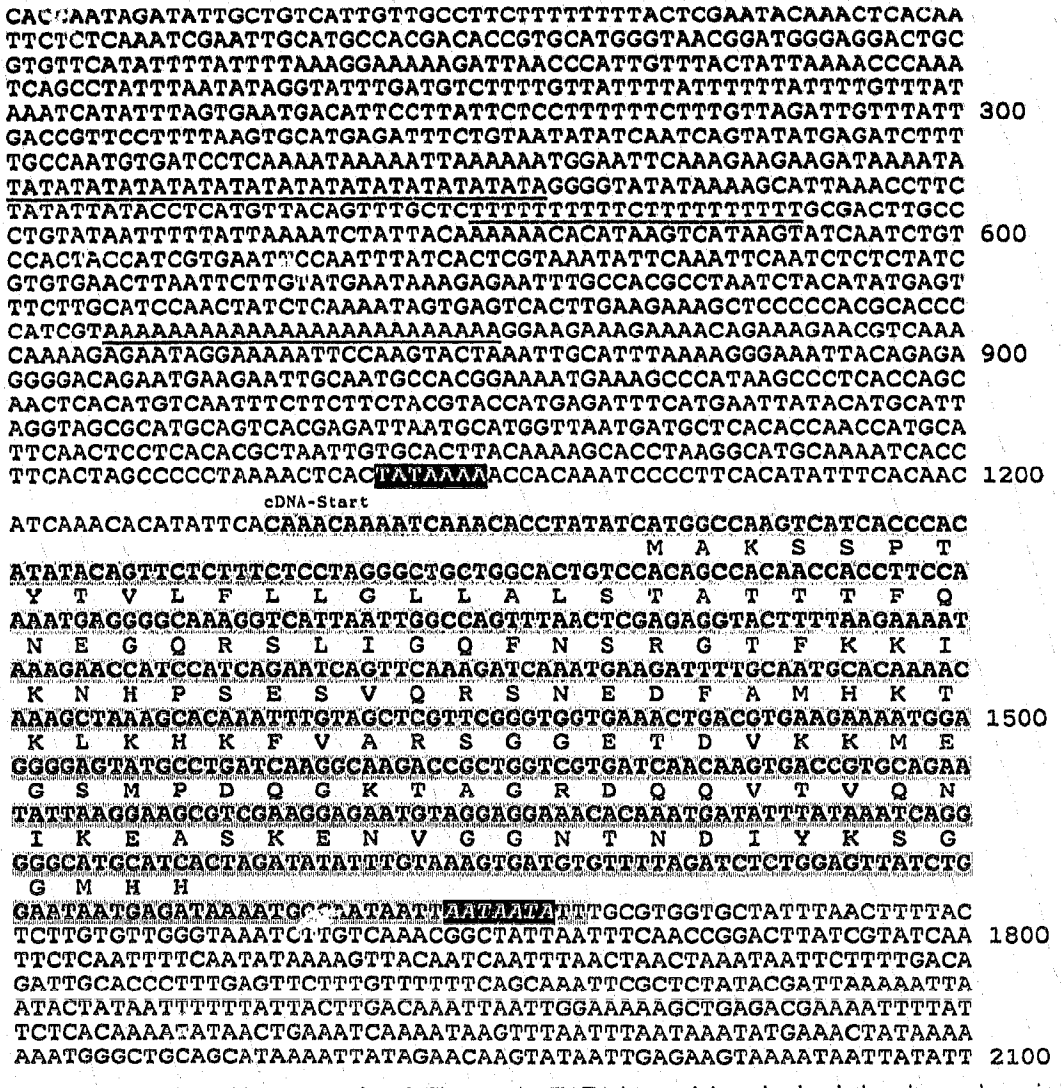

Fig. 1. Nucleotide and deduced amino acid sequence of *tap2*. The putative TATA-box and the polyadenylation site are shown in black boxes. The nucleotide sequence of the cDNA is shaded. Underlined are some striking AT-rich elements.

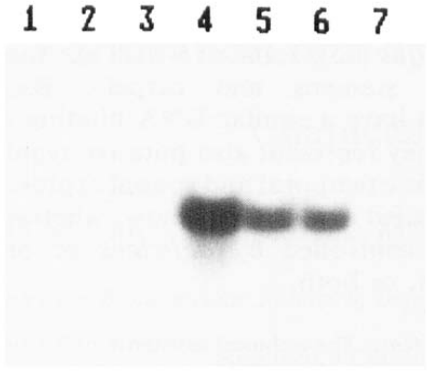

Fig. 2. Northern blot with poly(A)$^+$ mRNA isolated from different tissues and different developmental stages of wild type plants. 2 μg poly(A)$^+$ mRNA, twice purified on oligo d(T) cellulose, were loaded per lane. Radioactively labelled *tap2* cDNA was used as hybridization probe. Lane 1, leaf; 2, seedlings; 3, 2 mm inflorescences; 4, 5 mm inflorescences; 5, 5 mm flower buds; 6, 10 mm flower buds and 7, mature flower.

that it is secreted. The protein contains 24 basic and 12 acidic residues, and 51 of the total amino acids are glycine, serine, threonine or lysine. No homology to amino acid sequences of known proteins in the data banks was found in a search conducted with the deduced amino acid sequence of *TAP2*.

A comparison of the cDNA and genomic sequence revealed that the gene is not interrupted by introns (Fig. 1). In addition to the coding region, 1200 bp upstream of the cDNA start were sequenced. A putative TATA box is detected 50 bp upstream of the cDNA start. Several striking AT-rich motives of unknown function can be found further upstream. Southern blot analysis showed that only one copy of the *tap2* gene is present in the haploid genome of *A. majus* (data not shown).

Northern blot analysis with the *tap2* cDNA as radioactive probe revealed that the gene is expressed in developing flower buds but not in leaves and, as expected, not in the buds of the *def*^{all} mutant (Fig. 2). In Northern blots with mRNA from various developmen-

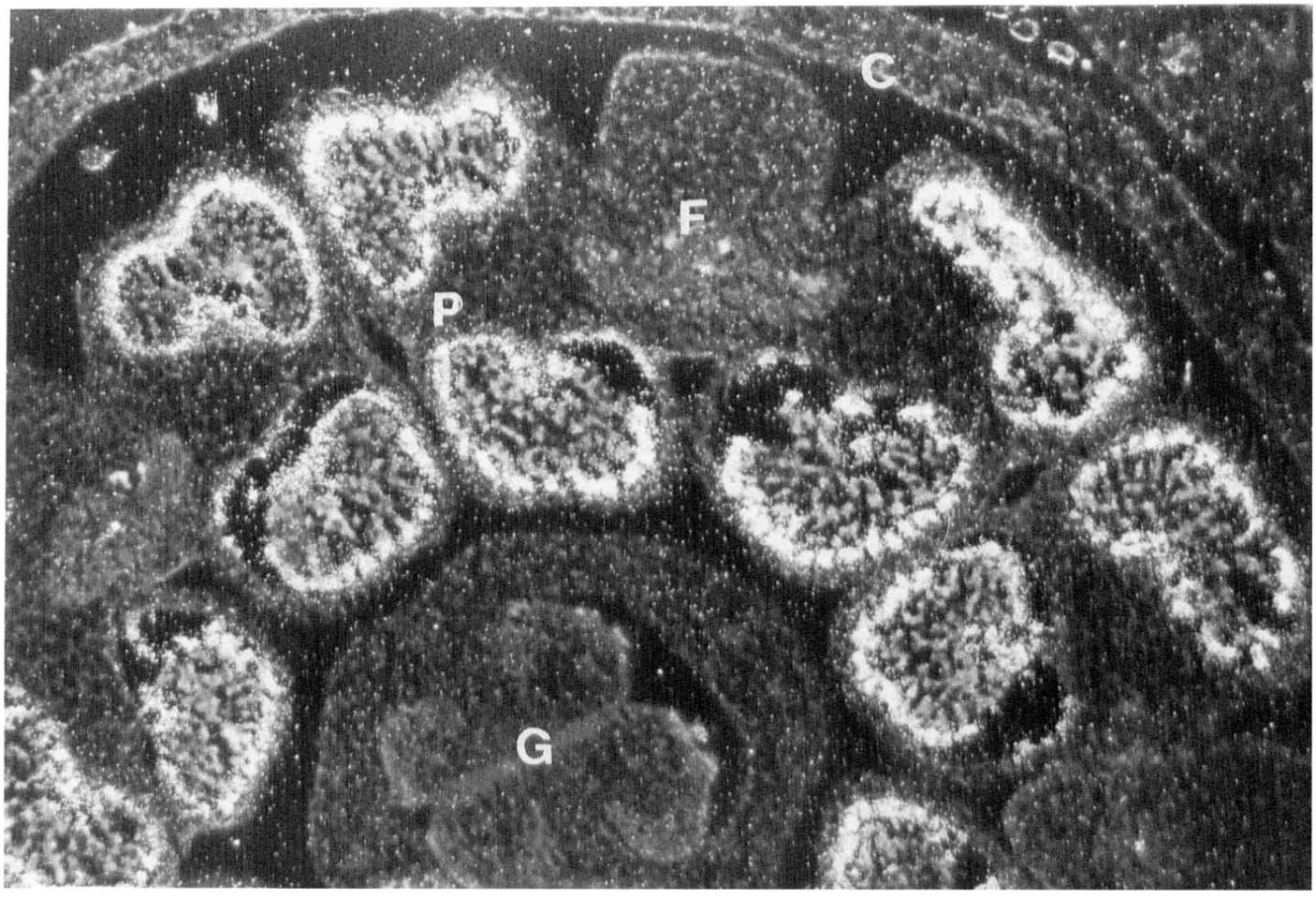

Fig. 3. In situ hybridization with labelled antisense RNA of the *tap2* cDNA. The picture shows a crossection through a young wild type flower bud of *A. majus*. The probe hybridizes strongly to the tapetal cells of the anthers. (c, corolla; f, filament; p, pollen sac; g, gynoecium.) No hybridization to the tapetal cells was observed with sense and antisense RNA from *deficiens*, a gene not expressed in the tapetum (data not shown), thus indicating that hybridization of the *tap2* antisense RNA is specific.

tal stages of the flower *tap2* transcript is detected in young inflorescences (5 mm in size), in 5 mm and 10 mm buds, but not in very early stages (2 mm inflorescences) and in mature flowers (Fig. 2). In situ hybridization experiments showed that a labelled antisense RNA of *tap2* hybridizes only with RNA of a single cell layer of the anthers, the tapetum (Fig. 3). Hybridization was not detectable in 2 mm inflorescences and in nearly mature anthers, where the tapetal cells had already started to disintegrate. The results of the in situ hybridizations are in agreement with the Northern experiment. Thus, *tap2* is a tapetum-specific gene expressed from the onset of tapetum cell differentiation until the degeneration of the tapetum in late developmental stages of the flower.

The tapetum is a physiologically very active tissue [12]. It is probably responsible for the nutrition of the developing pollen cells [13] and for the delivery of compounds that are deposited in the exine of the pollen [14]. Furthermore, the sporophytic tapetum cells have to communicate with the gametophytic pollen cells to coordinate the expression of genes necessary for normal pollen development. Since a putative signal peptide indicates that the *TAP2* protein may be secreted, *tap2* could be directly involved in one of the tapetal functions described above. The fact that a *tap2* probe hybridizes in Southern blots with genomic tobacco DNA (data not shown) indicates that this gene is conserved in other plant species.

Deficiens is predominantly expressed in the petals and in the filament of the stamen, but a basal level of expression is found in all floral organs (H. Sommer, unpublished data). Since the expression pattern of *tap2* differs from that of *deficiens* it seems questionable that *tap2* is a direct target gene of *deficiens*, although the basal level of *def* expression may be still sufficient to control *tap2* expression. Recently several other genes, encoding putative transcription factors with homology to the DNA-binding domain of *deficiens*, were isolated from *A. majus* [15], some of which are specifically expressed in stamens and carpels. Because these homologues have a similar DNA binding domain like *deficiens*, they represent also putative regulatory genes for the specific temporal and spatial expression of *tap2*. A more detailed analysis will show, whether expression of *tap2* is controlled by *deficiens* or one of these homologues, or both.

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