Distal regulatory regions restrict the expression of *cis*-linked genes to the tapetal cells

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Abstract The oleosin glycine-rich protein genes *Atgrp-6*, *Atgrp-7*, and *Atgrp-8* occur in clusters in the *Arabidopsis* genome and are expressed specifically in the tapetum cells. The *cis*-regulatory regions involved in the tissue-specific gene expression were investigated by fusing different segments of the gene cluster to the *uidA* reporter gene. Common distal regulatory regions were identified that coordinate expression of the sequential genes. At least two of these genes were regulated spatially by proximal and distal sequences. The *cis*-acting elements (122 bp upstream of the transcriptional start point) drive the *uidA* expression to floral tissues, whereas distal 5′ upstream regions restrict the gene activity to tapetal cells. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

The search for *cis*-acting elements that regulate gene expression in plants has been restricted mainly to single gene analyses. Such an approach holds back the identification of regulatory elements involved in the coordinated expression of several genes. Nevertheless, multigene expression regulation strategies with shared *cis* elements are highly predictable and have already been described in other organisms. The best known example is the human β-globin gene cluster, in which the tissue- and development-specific expression of individual globin genes is governed by the direct physical interactions between the globin promoters and the so-called β-locus control region (β-LCR), which are mediated through binding of tissue-restricted and ubiquitous transcription factors. β-LCR is not only responsible for the erythroid-specific expression pattern of β-globin genes but also supposedly involved in

chromatin structure remodelling [1,2]. LCRs may allow chromosomal position-independent expression, overcoming the heterochromatin-mediated silencing associated with centromeric locations of genes or *loci* [3].

We have previously reported that the oleosin glycine-rich protein genes are organized in tandem in the *Arabidopsis thaliana* genome and their expression is coordinately regulated in a developmental- and spatial-specific manner [4,5]. Hence, it is attractive to consider that LCR-like regions may be present in the oleosin glycine-rich protein gene cluster ensuring that their products are synthesized at the right time in the appropriate place.

These genes belong to a complex group of proteins whose major structural feature stands for the presence of a great proportion of glycine residues in their primary structure, thus designated glycine-rich proteins (GRPs) [6]. In spite of the great number of isolated GRP genes, the roles of most of their encoded proteins are not yet well defined. GRPs may have very diverse subcellular localizations, and the occurrence of other motifs in their primary structure, such as RNA binding consensus sequences, amino-terminal signal peptide, oleosin conserved domain, and CCHC zinc fingers among others, claim for different biological functions [6,7].

Atgrp-6-, *Atgrp-7*-, and *Atgrp-8*-encoded proteins fit in the structural characteristics of the pollenins, i.e. an oleosin-like domain at the N-terminus followed by a C-terminus that often contains motifs characteristic of structural proteins [8]. The genes are exclusively expressed in the tapetum of developing anthers, at a stage characterized by a reduced general secretory activity and by an increased lipid deposition. The lipid accumulation has been observed in the plastoglobuli and in a novel cellular organelle that we designated lipid protein body (LPB), because it is rich in basic proteins and unsaturated lipids [5]. A similar structure was identified in *Brassica* [9] and designated tapetosome. Full-length pollenins have been proposed to associate with tapetal lipid bodies via their oleosin-like domains. When the tapetals cells undergo apoptosis, the tapetosomes are released from the cells, come into contact with proteases that remove oleosin-like domains, and leave the mature pollenins to function as the major protein components of the pollen coat [8]. The accumulation of AtGRP-6, AtGRP-7, and AtGRP-8 transcripts has been correlated with the increase of the LPBs (or tapetosomes) in the tapetum [5].

We evaluated the expression pattern of the *Atgrp-7* and

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Atgrp-8 genes driven by 5′ upstream overlapping genomic segments, which were assayed by the *uidA* reporter gene. Two distal regions proved essential to restrict the reporter gene expression to the tapetum. Their absence results in the misexpression of the reporter gene in the style, the longitudinal axis of the ovary, and the flower peduncle. The negative control of *cis*-linked genes driven by shared silencers and the role of high-order chromatin organization are discussed.

2. Materials and methods

2.1. Atgrp genomic constructs

The positions of the restriction sites are related to the 10G1 genomic clone (accession number Z11858) described previously [4]. A *Nco*I site was introduced into the start codon region of the *Atgrp-7* and *Atgrp-8* genes by site-directed mutagenesis. Fragments of approximately 1.5 kb 5′ upstream of the coding sequences of the two genes were obtained by *Bam*HI (at position 3448 bp)/*Nco*I and *Bal*I (at position 6741 bp)/*Nco*I digestions, for *Atgrp-7* and *Atgrp-8* respectively. The fragments were fused to the *uidA* coding sequence, with the octopine synthase (ocs) 3′ end, in the vector pGUSCRS1 [10]. These chimeric genes were cloned as *Bam*HI/*Bam*HI and *Hin*dIII/*Bam*HI fragments into the plant transformation vector pTC021 (a derivative of the pGSC1700 vector [11]), providing constructs 1B and 1A, respectively (Fig. 1).

The *Atgrp-8* promoter region, preceded by the coding sequence of *Atgrp-7* and its putative promoter, was obtained as a *Nhe*I (at position 3556 bp)/*Nco*I fragment, and fused to the *gus* reporter gene in the vector pGUSCRS1. The chimeric gene was isolated as a *Hin*dIII/*Bam*HI fragment and cloned into the plant transformation vector pTC021, giving rise to construct 2A (Fig. 1).

Constructs 2B and 2C (Fig. 1) were obtained by digesting the genomic clone with *Hin*dIII (a vector site at the end of the *Arabidopsis* genomic sequence in the clone 10G1) and *Nco*I. The whole 5′ upstream genomic sequence preceding *Atgrp-8* and *Atgrp-7* was fused to the *gus* reporter gene in the vector pGUSCRS1 and cloned as a *Hin*dIII/*Bst*EII fragment into the plant transformation vector pTC021.

2.2. 5′ Deletions in construct 2C

Deletion 3A (Fig. 1) was generated by polymerase chain reaction (PCR) using primers GTTCCGGCGTCT<u>AAGCTT</u>GCAGAAT-CGGGG (1) and CTCCTAGGGCAA<u>CTGCAG</u>CGGTAGCCCCTC (2). Primer 1 introduced a *Hin*dIII site at the *AtOlnB;1* stop codon. The 1600-bp PCR-derived fragment, containing the promoter and the first exon of *Atgrp-6*, was digested with *Hin*dIII and *Pst*I and used to substitute the entire region upstream of the *Pst*I site (position 2489 bp) in construct 2C.

Deletion 3B (Fig. 1) was constructed using a 243-bp PCR-derived fragment generated with primers AGATCAAACGTAACTCA-C<u>AAGCTT</u>CTCTCG (3) and 2. Primer 3 introduced a *Hin*dIII site at the *Atgrp-6* start codon. The PCR-derived fragment containing the first exon of *Atgrp-6* was digested with *Hin*dIII and *Pst*I and used to substitute the whole *Pst*I upstream region in construct 2C.

Deletion 3C (Fig. 1) was generated by PCR with primers CTTTGGCTCTTT<u>AAGCTT</u>AATTTATTTATT (4) and CTCC-AATCCTAT<u>GTCTAC</u>ATCACACACACA (5). Primer 4 introduced a *Hin*dIII site at the 5′ splice site of *Atgrp-6*. The 287-bp PCR-derived fragment, containing the *Atgrp-6* intron, was digested with *Hin*dIII and *Acc*I and used to substitute the region upstream from the *Acc*I site (position 2794 bp) in construct 2C.

A 3707-bp fragment containing the whole *AtOlnB-1* as well as the promoter, exon 1, and intron of *Atgrp-6*, was deleted from construct 2C by digestion with *Acc*I followed by plasmid religation, generating construct 3D (Fig. 1).

2.3. 5′ Deletions in Atgrp-7 promoter region

Deletion 4A (Fig. 1) was obtained by digesting construct 1B with *Bst*XI (at position 4180 bp) and religation, deleting a fragment of 732 bp from the uncoding region 5′ upstream of *Atgrp-7*.

The *Bam*HI fragment used to generate construct 1B was subcloned in vector pUC19 (New England Biolabs, Beverly, MA, USA). The resulting plasmid was digested with *Bam*HI and partially digested

with *Bgl*II (at positions 4506 bp and 4802 bp, respectively), removing two fragments of 1058 bp and 1354 bp 5′ upstream of *Atgrp-7*. The remaining *Bam*HI/*Bgl*II fragments were cloned into the plant transformation vector pTC021 giving rise to constructs 4B and 4C, respectively (Fig. 1).

2.4. Plant growth conditions

Seeds of *A. thaliana* (L.) Heynh. ecotype C24 (Lehle Seeds, Round Rock, TX, USA) were grown in vitro, on GM medium, under a 16-h photoperiod at $22 \pm 2°C$ [12]. *Nicotiana tabacum* Petit Havana SR1 and *Brassica napus* cv. Drakkar were grown in MS medium [13] under the same culture conditions as for *A. thaliana*. Tobacco and *Brassica* transgenic lines were selected by germination on MS medium supplemented with 50 mg/l kanamycin. Transformed plants were grown on soil under normal greenhouse conditions. Transgenic lines were selected by germination on GM medium supplemented with 50 mg/l kanamycin. Flowers and seeds were obtained from transgenic lines grown in soil in a phytotron chamber.

2.5. Plant transformation

Lines of transgenic *A. thaliana* ecotype C24 carrying each construct were established with *Agrobacterium tumefaciens*-mediated transformation [12,14]. All binary vectors containing the *Atgrp* promoter–*uidA* chimeric fusions and deletions were mobilized by the helper plasmid pRK2013 into *A. tumefaciens* C58C1 harboring the pMP90 plasmid [15]. *B. napus* cv. Drakkar [16] and *N. tabacum* Petit Havana SR1 [17] were transformed and regenerated as described. The insert copy number was assessed by analysis of kanamycin resistance segregation. At least five homozygous independent transformants were selected from each construct for *gus* expression analysis.

2.6. Histochemical GUS assays and microscopic analysis of plant material

Histochemical GUS assays of plant tissues were carried out according to Jefferson et al. [18], with minor modifications. After maximum 6 h of reaction, plants were fixed in 2.5% glutaraldehyde in 100 mM NaPO$_4$ (pH 7.0) at 4°C for 16 h. Before being photographed under the stereomicroscope (Stemi SV11; Zeiss, Jena, Germany) the material was incubated in 70% ethanol, at 4°C, for at least 18 h to remove chlorophyll. For Nomarski images, tissues were cleared as described [19].

GUS-stained tobacco and *Arabidopsis* flowers at different developmental stages were fixed in 2.5% glutaraldehyde in 100 mM NaPO$_4$ (pH 7.0) at 4°C for 16 h, dehydrated through an ethanol series, and embedded in paraffin. Serial sections (40 µm) of plant material were attached to coated microscope slides and mounted with Depex mounting medium (Gurr, Poole, UK). Sections were photographed under a light microscope (Diaplan, Leitz, Germany).

B. napus tissues were assayed with GUS according to De Block and Debrouwer [20].

Pollen grains were germinated in medium containing 17% sucrose, 30 mg/l CaCl$_2$·2H$_2$O, 100 mg/l H$_3$BO$_3$, and 0.6% agarose, pH 6.4, at 28°C. After 4 h, aliquots were removed and GUS-stained.

3. Results

3.1. Atgrp-7 and Atgrp-8 promoters do not limit uidA expression to the tapetum

Previously in situ hybridization studies showed that *Atgrp-7* and *Atgrp-8* gene expression was restricted to the tapetum cell layer [5]. To characterize the *cis*-acting regulatory regions of these genes, promoter–*gus* fusions were made and transgenic plants were generated (Fig. 1, constructs 1A and B). Histochemical analysis of the transgenic plants revealed that GUS activity was not restricted to the tapetum cell layer of the anther. The reporter gene expression was also seen in the style, ovary vascular tissues, anther filaments, and in the peduncle (Fig. 2A). The same expression pattern was observed for all independent lines analyzed. The *uidA* expression was limited to anther stages 2 and 3, as observed previously for the endogenous *Atgrp-7* and *Atgrp-8* genes [5].

Fig. 1. Schematic representation of *Atgrp* genomic constructs and *gus* expression profile. Black boxes represent the exons from the clustered genes in the clone 10G1 (accession number Z11858). *Arabidopsis* flower illustrations I, II, and III summarize the three distinct *gus* profiles (in light gray) observed in the transgenic plants. The GUS activity in the different tissues is represented by +++ (strong), + (weak), and − (no activity). The figures −744, −418, and −122 refer to the remaining bp 5′ upstream from the transcription start site of *Atgrp-7* gene in constructs 4A, 4B, and 4C, respectively.

3.2. Atgrp-7 and Atgrp-8 are regulated by shared distal regulatory regions

To investigate whether these genes are coordinately regulated by a common mechanism, different fragments of the *Atgrp* gene cluster were fused to the *uidA* reporter gene (Fig. 1). The first construct, in which the genomic fragment from *Atgrp-7* 5′ upstream of the untranscribed region to the *Atgrp-8* promoter that controlled reporter gene expression (Fig. 1, construct 2A), produced a pattern identical to that of the *Atgrp-7* and *Atgrp-8* promoters alone (constructs 1A and 1B).

Interestingly, when *uidA* was fused to the whole 10G1 genomic fragment 5′ upstream of the *Atgrp-8* translated sequence (Fig. 1, construct 2B) GUS activity was only detected in anthers (Fig. 2B). The same result was obtained with the construct 2C, in which the *uidA* reporter gene is controlled by the 10G1 genomic fragment 5′ upstream of the *Atgrp-7* translated sequence (Fig. 1). Anther sections of these plants showed *uidA* expression specifically to the tapetum cell layer. No GUS activity was found in any other flower tissue (Fig. 2C). The same expression pattern was observed in all independent lines analyzed. The GUS staining of the whole inflorescence of *Arabidopsis* transformed with the tapetum-specific constructs 2B and 2C was detected only in flower buds ranging from 0.5 mm to 1.5 mm (Fig. 2D). These results confirmed in situ hybridization analysis of *Atgrp-6*, *Atgrp-7*, and *Atgrp-8*, in which transcripts could only be detected in anthers at developmental stages 2 and 3 [5].

The results indicated that the region extending from the 10G1 5′ border to the end of the *Atgrp-6* coding sequence contained regulatory elements necessary for the tapetum-specific expression of *Atgrp-7* and *Atgrp-8*. To refine these data, four sequential deletions upstream of the *Atgrp-7* promoter were fused to *uidA* and transferred to *Arabidopsis*. Histochemical analysis showed that construct 3A, in which the sequence from the 5′ border of 10G1 to the end of the *AtOlnB-1* coding sequence was deleted, drove the *gus* expression to the edge of the style's vascular bundle as well as to the tapetum (Fig. 2E). The same *gus* expression pattern was observed in plants transformed with the three subsequent constructions, in which the 5′ upstream untranslated region, exon 1, and intron of *Atgrp-6* were deleted (Fig. 1, constructs 3B, 3C, and 3D, respectively).

3.3. Proximal 5′ 122 bp program Atgrp-7 gene expression in flower tissues

The *gus* expression pattern obtained with constructs 1A and 1B indicated that each promoter region contained *cis*-acting elements that drive the expression of *uidA* to the tapetum, style, ovary, and flower peduncle. However, in endogenous *Atgrp* genes, the 'out-of-tapetum' *cis*-acting elements seem to be inaccessible to transcription factors. In an attempt to localize these elements in the *Atgrp-7* promoter, deletions at positions −744, −418, and −122 were generated (Fig. 1, constructs 4A, 4B, and 4C, respectively). Histochemical analysis
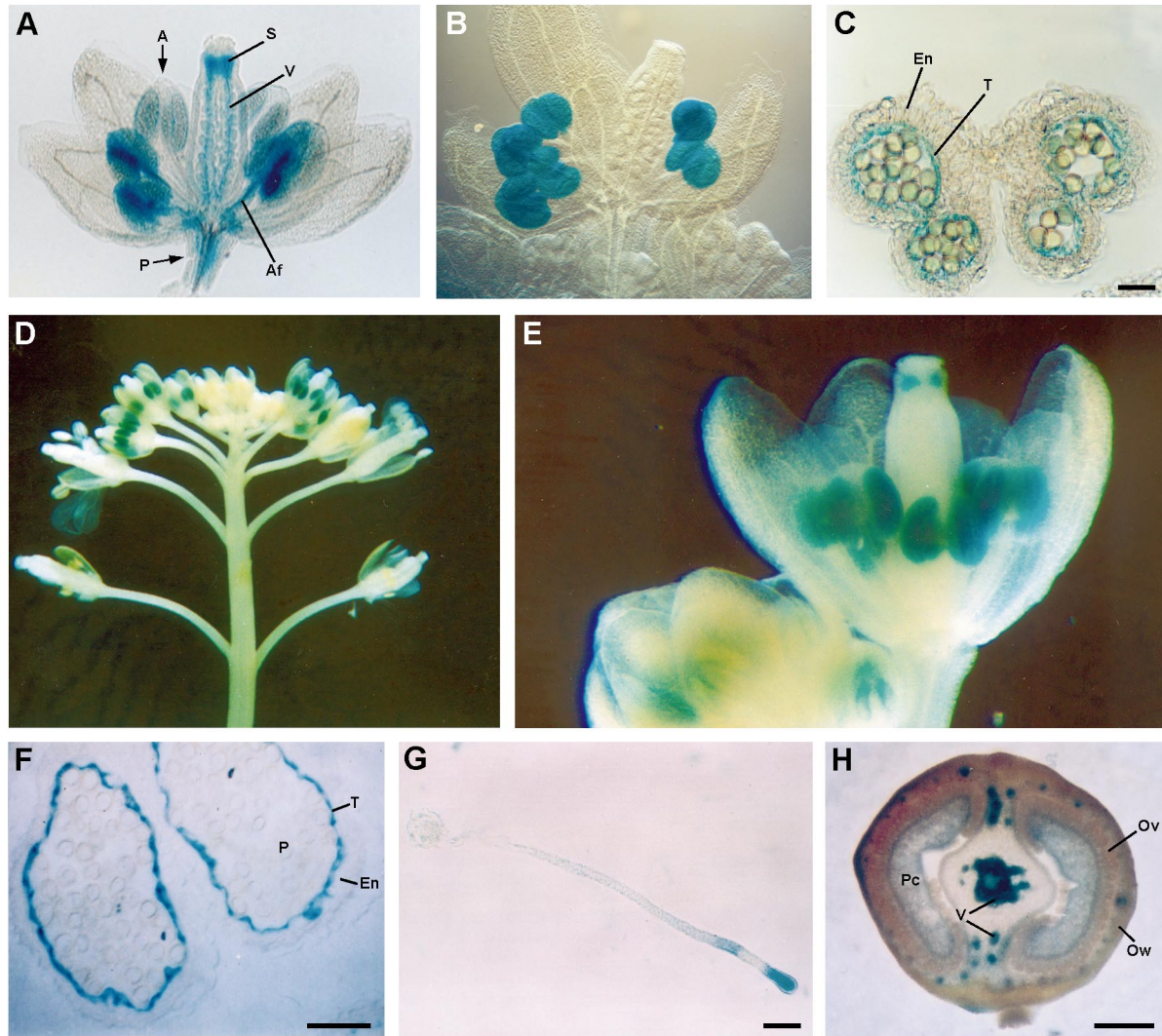
Fig. 2. *gus* tissue expression pattern in flowers of *Arabidopsis*, *Brassica*, and tobacco transformed with *Atgrp–uidA* constructs. A: *Arabidopsis* flower with *gus* expression profile I (see Fig. 1). B: *Arabidopsis* flower with anther-specific expression of the *uidA* reporter gene (expression profile II). C: Transverse section of an *Arabidopsis* flower showing *gus* expression restricted to the tapetum layer (expression profile II). D: Inflorescence of *Arabidopsis* showing the developmental regulation of *gus* expression (expression profile II). E: *Arabidopsis* flower showing *gus* expression profile III. F: *B. napus* plants transformed with construct 2B or 2C with *gus* expression restricted to the tapetum layer. GUS-stained transverse section of a transgenic anther. G,H: Germinating pollen grain (G) and transverse section of the ovary (H) showing *gus* expression in the vascular tissue of transgenic tobacco harboring construct 2B or 2C. A, anther; Af, anther filament; En, endothecium; Ov, ovary; Ow, ovary wall; P, peduncle; Pc, placenta; S, style; T, tapetum; V, ovary vascular tissue. Bars = 40 μm (C), 50 μm (F), 100 μm (G), 250 μm (H).

of the transgenic plants showed that the 122 bp upstream of the *Atgrp-7* transcription start site are enough to drive *uidA* expression to all the above mentioned tissues. The expression pattern was identical to that shown in Fig. 2A for all independent lines analyzed.

Comparing these data with those obtained with constructs 2B, 3A, 3D, and 1B, three distinct regions have to contain elements that regulate the *Atgrp-7* spatial expression pattern: (i) from −4925 to −4010, comprising the *AtOlnB-1* gene plus 300 bp 5′ upstream, (ii) from −2128 to −1470, comprising the *Atgrp-6* second exon plus 100 bp 3′ downstream, and (iii) from −122 to +1, comprising *Atgrp-7*'s own promoter. The absence of the first region disrupted tapetum-specific *uidA* expression and drove it also to the style's vascular bundle. By deleting the second region, the 'out-of-tapetum' *uidA* expression was expanded to the whole style, ovary vascular tissues, anther filament, and peduncle. The distally negative-reg-

ulatory regions are probably also involved in restricting *Atgrp-8* expression to the tapetum cells (compare results obtained with constructs 2A and 2B).

3.4. Constructs 2B and 2C in heterologous systems

The *Arabidopsis* tapetum-specifically expressed gene fusions 2B and 2C were also stably introduced into *B. napus* and *N. tabacum*. As expected from their close phylogenetic relationship, the same tissue-specific expression pattern was observed in *Arabidopsis* and *Brassica*. Anther sections confirmed that the *Atgrp-7* and *Atgrp-8* promoters, combined with the remaining genomic fragment, were active in the tapetum cell layer (Fig. 2F). The *gus* expression in the tapetum persisted until flower buds reached 5 mm length and decayed as they increased. This switch-off coincided with the degeneration of the tapetal cell layer.

Constructs 2B and 2C were unable to drive the same tissue-

specific expression in transgenic tobacco plants. GUS activity accumulated in two distinct flower tissues: post-mitotic pollen grains (Fig. 2G) and vascular tissues of the ovary (Fig. 2H) as well as in the vascular tissues of the peduncle and petals (data not shown).

4. Discussion

Here we describe the coordinated spatial and temporal expression of two *Arabidopsis* pollenin genes (*Atgrp-7* and *Atgrp-8*) and find distal regulatory regions that control the tissue-specific expression of these genes. Although their tapetal expression has been characterized previously by in situ hybridization analysis [5], the genomic constructs indicate that several regulatory elements are required for the accomplishment of this expression pattern. *Atgrp-7* promoter–*uidA* fusions showed that, besides the tapetum *cis*-acting elements present in the first 122 bp 5′ upstream of the transcription initiation site, two different regions outside the gene should contain regulatory elements responsible for restricting its expression to the tapetal cells. One of these regions, located at position 1–901 of clone 10G1 (accession number Z11858), comprises the whole *AtOlnB-1* gene plus 300 bp 5′ upstream, and inhibits the expression on the style's vascular tissue. A second region has been identified between 2797 and 3450 and comprises the second exon of *Atgrp-6* gene plus 100 bp downstream. The absence of this sequence in *Atgrp-7* and *Atgrp-8* promoter–*uidA* fusions causes the misexpression of *gus* in other floral tissues (style, ovary vascular tissue, anther filament, and peduncle). Altogether, these results indicate, for the first time in plants, that the coordinated spatial expression of sequential genes may be regulated by shared distal elements.

It is important to note that, although the spatial expression pattern depends on regulatory elements located 5′ upstream of the *Atgrp* genes, the temporal expression does not change. The distinct floral expression patterns assayed by GUS activity can only be observed in flower buds of 0.5 to 1.5 mm, in which the endogenous *Atgrp* genes are active in the tapetum. Thus, the availability of transcription factors in the other floral tissues in which GUS activity has been detected is also temporally regulated and restricted to a certain developmental stage.

Comparison of the proximal 5′ upstream sequences of each *Atgrp* gene and of *AtOlnB-1* revealed five conserved motifs [4]. Deletion analysis of the *Atgrp-7* promoter indicates that the *cis*-acting sequences responsible for tapetum, style, ovary vascular tissue, anther filament and peduncle expression are located within −122 to +1, where only motifs I, II, and III are present. These motifs are hot candidates for the binding of *trans*-activating factors, responsible for the flower-preferential expression pattern of the *Atgrp-7* gene. The same motifs are found 5′ upstream of every coding sequence in the cluster, in which they might play the same regulatory role. When the sequence is compared with other anther- and/or tapetum-specific promoters, such as the tobacco TA29 [21], the *Brassica* and *Arabidopsis* A9 [22,23] and the *Brassica BnOlnB;3* and *BnOlnB;4* [24,25], no significant homology is found (data not shown), corroborating the hypothesis of the existence of different networks that regulate spatial expression patterns.

The full-length genomic constructs 2B and 2C (Fig. 1) produce the same *gus* expression pattern in *Brassica*, indicating that all the *cis*-acting elements and transcriptional factors responsible for the tapetal-specific expression are conserved

within this family. In contrast, in tobacco, the same constructs drive *gus* expression to pollen, vascular tissues of the ovary, receptacle, peduncle and petals. These results indicate that although *Arabidopsis cis*-acting regulatory regions can be recognized by tobacco transcription factors, they do not reproduce the tissue-specific expression observed in Brassicaceae.

Genomic segments ranging from 0.5 to 2 kb 5′ upstream of the transcription start site are commonly accepted to be sufficient to reproduce the correct tissue expression pattern of an endogenous gene [26,27]. Nevertheless, the involvement of regulatory sequences outside the so-called promoter region was also verified. Larkin et al. [28] demonstrated that, in addition to the promoter, elements present in the 3′ untranslated region of the *Arabidopsis GLABROUS 1* gene are also essential for its expression in the trichome initiation sites. Regulatory elements located in the intron, 3′, and 5′ flanking regions of the *sus3* potato gene are necessary to drive high expression levels of the reporter gene in a tissue-specific manner [29]. Another example is the intragenically located regions necessary to confer correct tissue-specific expression pattern of the *AGAMOUS* gene [30,31]. The presence of distal sequences that regulate the expression of genes has been described before, as in the cytochrome P-450 3A4 promoter where a potent enhancer module has been identified 8 kb distal to the transcription start point [32]. Although regulatory elements frequently have a positive effect on the transcription rates, in some cases tissue-specific expression can be achieved through negative factors [33–36]. Silencers are proposed to have a modular organization, like the promoters and enhancers, which are responsible for the repression of the transcription independently of their position or distance from the transcription initiation sites [37].

Here, we have shown that distal elements are essential for the correct spatial expression of at least two sequential genes, namely *Atgrp-7* and *Atgrp-8*. These elements act as silencers for both genes, characterizing a coordinated gene expression regulation. The results are consistent with a model in which positive and negative regulatory elements are required for the accomplishment of the expression pattern of *Atgrp-7* and *Atgrp-8*. Distal silencers would inhibit proximal positive *cis*-acting elements responsible for the 'out-of-tapetum' gene expression. Fusions of the distal negative regulatory regions with the non-restrictive constructs (1B, 1A, and the deletions on the *Atgrp-7* promoter), preserving their relative position in the genome by the insertion of an heterologous non-related sequence, will allow the validation of the proposed model.

Chromatin structure may also play a role in the coordinated expression of the clustered pollenin genes. Recent advances indicate that the chromatin structural organization is a critical parameter in gene expression regulation [38]. Although the high-order structures are not well defined yet, the genome seems to be subdivided in boundaries that limit the regulatory effects of positive and negative elements, such as enhancers and repressors [39]. Scaffold/matrix attachment elements (S/MARs) are candidates to contribute to the higher-order regulation of transcription by defining boundaries of independently controlled chromatin domains. Indeed S/MAR regions have been used to standardize transgene expression in plants [40,41]. To see whether the expression pattern observed with constructs 2B and 2C is conditional for their integration into the *Arabidopsis* genome, we have performed transient expression assays in intact flower tissues [42]. Although preliminary,

the results obtained with the tapetum-specific constructs showed that, while the temporal regulation is maintained, the spatial expression has been modified. GUS activity has also been found in the vascular bundles of the ovary and peduncle of flower buds ranging from 0.5 mm to 1.5 mm (data not shown). Therefore, we propose that the negative regulation of the *Atgrp* genes depends on a high-order chromatin organization, only obtained when the constructs are stably integrated into the *Arabidopsis* genome, as previously observed for other chromatin-regulated transgenes [41,43].

The eukaryotic consensus bipartite element of S/MARs [44] has been spotted twice in the genomic region spanning the pollenin cluster, 392 bp and 125 bp downstream of the coding regions of *Atgrp-6* and *Atgrp-7*, respectively. However, their deletion did not affect the spatial or temporal *gus* expression pattern, therefore they are not involved in restricting the expression of the pollenin genes to the tapetum (compare the results of construct 2A with 1A, and those of 1B with 4A).

The pollenin gene cluster is located in chromosome V and covers approximately 13 kb of the *Arabidopsis* genome. In silico analysis of this region shows that, besides the four genes already described, two additional genes have been found, an oleosin gene 5′ upstream of *AtOlnB-1* and an oleosin-GRP gene 3′ downstream of *Atgrp-8*. A second GRP cluster has been identified also at chromosome II. It contains the *Atgrp-3* gene [45] in tandem with three related *GRP* genes that have not been characterized molecularly yet. It is tempting to consider that these clusters, as well as several other structurally similar genes that are sequentially organized in the genome [46], could be coordinately regulated in a locus-controlled manner, this being one of the strategies adopted by eukaryotic organisms to achieve protein production at an appropriate amount, time and place.

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