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Arabidopsis monomeric G-proteins, markers of early and late events in cell differentiation

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ABSTRACT In *Schizosaccharomyces pombe*, septum formation is intricately controlled by proteins which constitute the SIN (Septum Initiation Network) signalling cascade. The SIN ensures the coordination between mitotic exit and cytokinesis. Yeast spg1p is a core component of the SIN pathway and we have previously characterized the two orthologs of this G-protein in *Arabidopsis thaliana* (named AtSGP1 and 2). In this work, the cell and tissue expression of *AtSGP* genes during plant development has been analysed using *AtSGP* promoter::GUS fusions in stably transformed *A. thaliana* lines. *AtSGP1* promoter activity was restricted to the quiescent centre, collumella cells, stomata guard cells and the stele while *AtSGP2* promoter activity was detected in atrichoblasts, trichomes and pollen. The observed promoter activities are in accordance with publicly available pollen, stomata guard cell and root transcriptome data. Two-hybrid experiments previously evidenced an interaction between AtMAP3Kepsilon1 and AtSGP1. The *AtMAP3Kepsilon1* promoter activity was detected in root apices, trichomes and ovule integuments. A genetic approach involving both markers of these specialized cells and mutant backgrounds was used to reinforce our hypothesis. It appears that, although highly conserved between plants and fungi, the spg1p G-protein has evolved in plants to perform a function different from the SIN pathway. Interestingly, cells expressing *AtSGPs* possessed limited or null mitotic activity. Our data suggests that AtSGP are crucial signalling components involved either in early cell fate specification, or in the final steps of cell differentiation. This is an interesting starting point for a wider study devoted to functional experiments designed to test these hypotheses.

KEY WORDS: *Arabidopsis thaliana, AtSGPs, atrichoblasts, pollen, quiescent centre*

Introduction

The development and growth of plant organisms is regulated by a series of intricate signalling pathways and transcriptional networks. They act to specify cell types and to maintain cell differentiation. During plant development, the implementation of the dynamic processes of cell division requires the integration and evaluation of signals coming from diverse cellular programs. Development integrates highly complex mechanisms. Cell specification in plants is determined through two different systems shared by animals and plants, cell position and cell lineage. The first one uses positional information, meaning that the development of a particular cell type is determined by its localisation relative to another cell type (Dolan, 2006). The establishment of a new cell type during development also uses a mechanism based on the control of an initial asymmetric cell division to drive either cell position or cell lineage (MacAlister *et al.,* 2007). To date, only a single signalling element, the receptor-like kinase SCRAMBLED, has been shown to function in cell type specification.

Previously, we identified and characterized several signalling components (kinases, G-proteins) in *Arabidopsis thaliana* that were closely related to the core components of the SIN (Septum Initiation Network) pathway of *Schizosaccharomyces pombe*. The SIN pathway controls events at the end of mitosis, coordinating mitotic exit and cytokinesis (Simanis, 2003). The G-protein

Abbreviations used in this paper: AtSGP, Arabdiposis thaliana G-protein; SIN, septum initiation network; QC, quiescent centre.

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AtSGP and the kinase *AtMAP3Kepsilon* are orthologues of the core components *spg1* and *cdc7* of the yeast SIN pathway (Champion *et al.,* 2004; Jouannic *et al.,* 2001). An original feature is that each of these SIN components possesses two paralogues in the *A. thaliana* genome, namely *AtSGP1* - *AtSGP2* and *AtMAP3Kepsilon1* - *AtMAP3Kepsilon2*. Overexpression of the *A. thaliana* genes *AtSGP1* and *AtSGP2* in yeast cells induced multiple rounds of septum formation without cell cleavage, similarly to their orthologue *spg1* (Champion *et al.,* 2004).

In *S. pombe*, spg1p and cdc7p interact both genetically and physically (Schmidt *et al.,* 1997). Assays in yeast showed an interaction between cdc7p and both AtSGP1 and AtSGP2. On the other hand no interaction could be detected between *Brassica napus* MAP3Kepsilon1 and spg1p, while BnMAP3Kepsilon1 could interact with AtSGP1, but this interaction was not detected with AtSGP2 (Champion *et al.,* 2004), a finding which supports the possibility of a functional difference.

Our previous results also revealed that, depending on the organs, *AtSGP1* showed higher expression (root) or reduced expression (flower) compared to its paralogue *AtSGP2* (Champion *et al.,* 2004). In this work, we further examined the possibility of *AtSGP* involvement in cell division or in cytokinesis, by carefully analysing the localization of *AtSGP1* and *AtSGP2* promoter activity using promoter::*GUS* constructs. Expression studies were performed in the reference Columbia ecotype and in various mutant backgrounds related to the cell types expressing each *AtSGP* genes. *AtSGP1* and *AtSGP2* promoter activity was observed in specialized cell types, with limited or null mitotic activity, including epidermal cells (atrichoblasts, trichomes, stomata guard cells), mature pollen, and the quiescent centre. Our data suggest that these GTPases act in early and late differentiation signalling mechanisms in several cell types.

Results

AtSGP1 *promoter activity in particular cell types*

Using transgenic *Arabidopsis thaliana* plants stably transformed with the *AtSGP1* promoter::β-glucuronidase (GUS) fusion construct, cell- and tissue-specific activity of the *AtSGP1* promoter was examined in planta. However, despite the previously reported *AtSGP1* expression at G2-M phases (Champion *et al.,* 2004), GUS staining appeared neither in the shoot apical meristem nor in the root meristematic zone containing mitotically active cells. In flowers, *AtSGP1* promoter activity was detected in the sepals, the style and the connective tissue of anthers (Fig. 1A). Furthermore, *AtSGP1* promoter activity was observed in very specialized leaf cells, the stomata guard cells (Fig. 1C), but GUS coloration was not seen at an earlier developmental stage in guard cell mother cells, nor in the meristemoid cells. *AtSGP1* promoter activity was found in stomata guard cells on both the abaxial and adaxial leaf epidermis. In addition, GUS staining was detected in young leaf hydathodes (Fig. 1B). Hydathodes are often found at the leaf margin, at the end of vascular bundles. They are specialised structures involved in secretion or water exudation.

Further histochemical analysis using ten day-old seedlings showed that the *AtSGP1* promoter directed expression of the GUS reporter gene particularly in the root tip of both primary and lateral roots. Indeed, the *AtSGP1* promoter::*GUS* fusion conferred an important expression in the QC cells (Fig. 1E). The QC consists of a four-cell organizing center that maintains stem

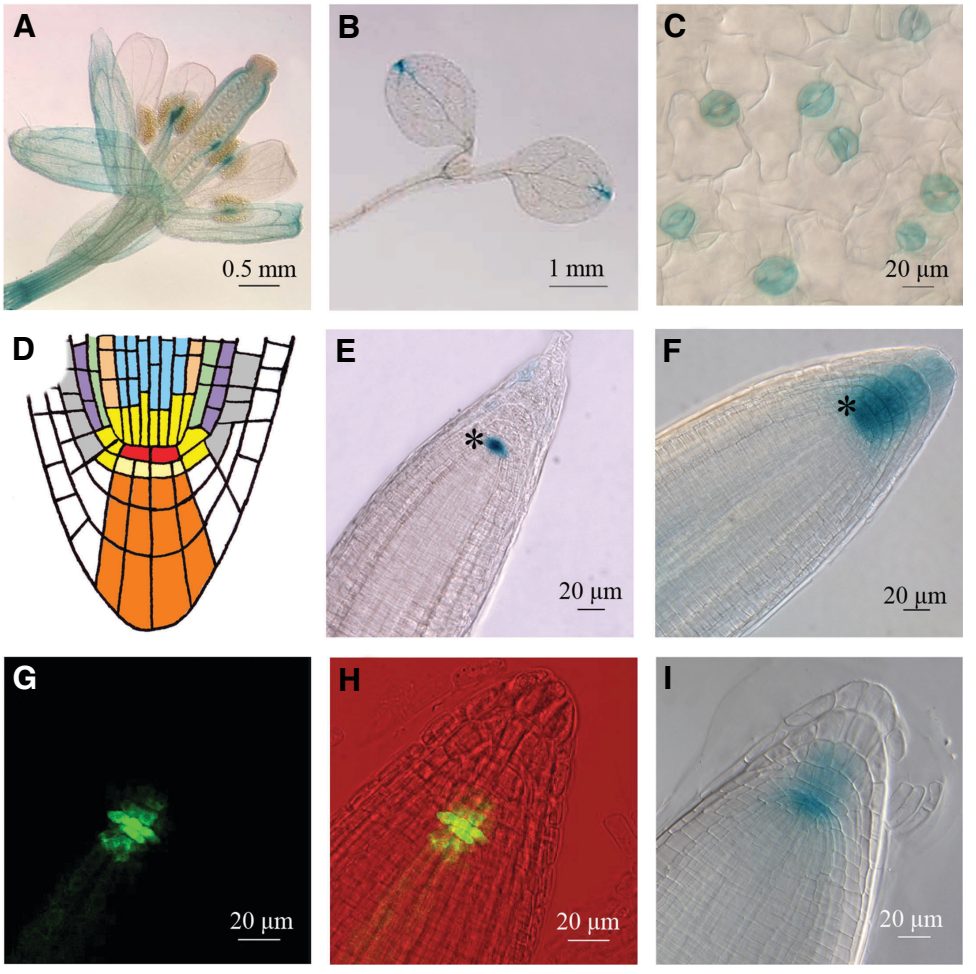

Fig. 1. *Arabidopsis thaliana AtSGP1* promoter activity patterns in transgenic T_2 lines. *The promoter activity is visualised by GUS staining of flower* **(A)**, *10 days old seedling* **(B)** *and leaf epidermis* **(C)**. *Note the staining in connectives (A), hydathodes (B) and stomata guard cells (C).* **(D)** *Colorized drawing of the root apical meristem region: gray: epidermis, purple: cortex, green: endodermis, peach: pericycle, blue: stele, white: lateral root cap, orange: columella, yellow: initials (stem cells), red: quiescent centre (QC).* **(E,F)** *GUS activity in the root apex of 12 day old seedlings. Depending on the different lines (e,f) GUS activity was restricted to the QC (marked with an asterisks *), or observed in QC, stem cells and columella cells.* **(G,H,I)** AtSGP1 *promoter activity in* DR5::GFP *lines. Both gene reporter activities colocalize at the QC.*

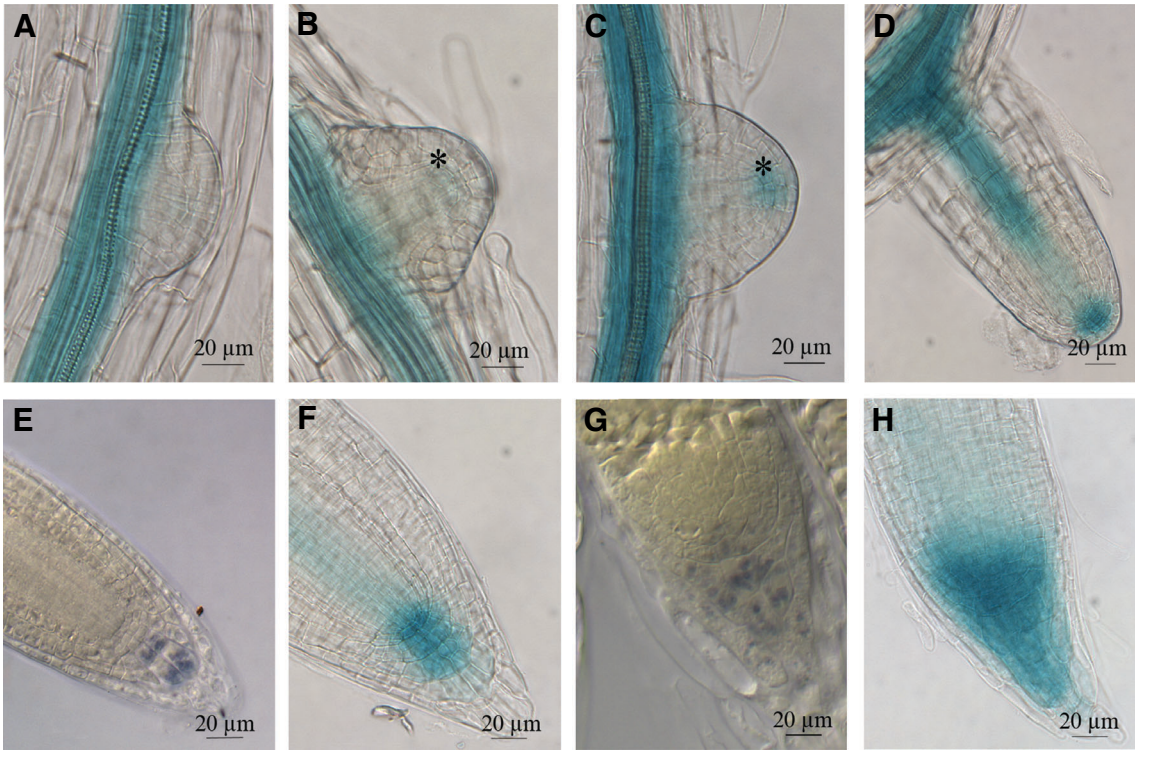

Fig. 2. *AtSGP1* promoter activity correlates with the positioning of the quiescent centre (QC). (A,D) *During initiation and development of the lateral root. QC is marked with an asterisk.* AtSGP1 *promoter activity is expanded in the* plt1 plt2 *double mutant background* **(G,H)** *compared to the wild type* **(E,F)**. *Starch granule staining marks differentiated columella cells* **(E,G)**.

cells mitotically active. Together the QC and the stem cells constitute the stem cell niche (Fig. 1D) (van den Berg *et al.,* 1997). A weaker expression was observed in cells (i.e. the columella initials, ground tissue or columella root cap) proximal to the QC, in most of the 20 lines tested (Fig. 1F). In order to ascertain the activity of the *AtSGP1* promoter in the QC, a plant line was generated that possessed both *AtSGP1* promoter*::GUS* and the auxin distribution marker *DR5::GFP*. Both reporter genes were assayed in the same roots. We first observed GFP fluorescence driven by the *DR5* promoter in the QC (Fig. 1G). Then, a histochemical analysis performed on the same root tips showed that GUS-expressing cells overlapped with the GFP-expressing cells (Fig. 1 H,I). The co-localization of the *AtSGP1* promoter activity (*ie* GUS staining) with the *DR5::GFP* reporter activation confirmed that *AtSGP1* promoter activity was present in the QC, and thus associated with high auxin concentrations (Ulmasov *et al.,* 1997).

GUS staining was monitored during lateral root initiation and development in order to determine at what stage the *AtSGP1* promoter activity was induced. No promoter activity was detected in early lateral root primordia emerging from pericycle cells (Fig. 2A). At further developmental stages, GUS staining was associated only with QC cells (Fig. 2 B,C,D). This suggests *AtSGP1* promoter activity to be associated with QC positioning.

To determine whether or not *AtSGP1* gene expression depended on QC-specific genes, we studied putative genetic interactions with *PLETHORA1* (*PLT1*) and *PLETHORA2* (*PLT2*) genes. Both *PLT* genes are required for specification and maintenance of the QC and stem cells in the root meristem (Aida *et al.,* 2004). In a *plt1-4 plt2-2* double mutant (mentioned thereafter as *plt1 plt2*), roots do not possess a functional QC, the columella contains an increased number of differentiated cells and its stratified structure is disturbed. Indeed, starch granules accumulated in all columella layers (compare Fig. 2 E,G), including cells at the position of the stem cells (Aida *et al.,* 2004). The root apical meristem is strongly modified in the *plt1 plt2* double mutant. We analysed the effect of the *plt1 plt2* mutations on *AtSGP1* promoter activity by introducing the *AtSGP1promoter::GUS* construct into the *plt1 plt2* double mutant. In the root meristem of T_2 transformed *plt1 plt2* lines, GUS staining expanded to at least two additional layers of differentiated columella cells containing starch granules, proximal to the mis-specified QC cells (compare Fig. 2 F,H). *AtSGP1* promoter activity was not suppressed in the *plt1 plt2* double mutant background, thus showing that *PLETHORA* is not required for *AtSGP1* promoter activity. Nevertheless, in the absence of *PLETHORA* expression both the QC fate and the initial cell fate are not specified, and the root meristem contains much more differentiated cells. These changes lead to a significantly enlarged *AtSGP1* promoter activity in the root meristem. Therefore, the expression domain of *AtSGP1* promoter activity depends on the *PLETHORA* genes.

About twenty transformed lines were found to exhibit a GUS expression pattern along root vascular tissues with weaker expression in mitotically active cells. Figure 2 A,B,C,D,F reveals *AtSGP1* promoter activity in the stele, at stages including differentiated pericycle and xylem cells. Weak staining was also detected in the stele at earlier stages upstream of the QC

(Fig. 2 D,F). Our data suggest a putative role for AtSGP1 in the differentiation of different stele cell types.

AtSGP2 *promoter activity is also detected in specialised cells*

Histochemical staining of the primary root of 10-day-old transgenic plants was performed on T1 and T2 plants transformed with a *AtSGP2promoter::GUS* construct. The localisation of GUS activity was totally different from that obtained with the promoter of its paralogue *AtSGP1*. It appeared neither in the apical meristem, nor in the QC. *AtSGP2* promoter activity in epidermal cells gradually increased in the upstream part of the division zone. The GUS staining was detected in differentiating cells near the onset of cell elongation (Fig. 3A). The staining was not homogenous, as files of stained cells were separated by files of unstained cells (Fig. 3 A,B). The *AtSGP2* promoter activity was never observed in cell files within the maturation zone. To determine more precisely the location of stained cells, transverse sections were performed from stained and embedded elongation zones of primary roots. The blue staining was limited to those epidermal cells located over tangential cortical cell walls (*i.e.* at the position of differentiating atrichoblast cells) (Fig. 3B insert), while no staining was detected in epidermal cells located over the junction of cortical cells, at the position of the differentiating trichoblasts (*i.e.* hair cells).

To determine whether or not the *AtSGP2* promoter activity is required to ensure that cells located in particular positions of the *A. thaliana* root epidermis differentiate into atrichoblast epidermal cells, the *AtSGP2* promoter activity was examined in a mutant background. In the *SCRAMBLED* (*SCM*) mutant *scm-2*, the *GLABRA2* promoter activity displayed a patchy distribution in the root epidermis (Kwak *et al.,* 2005). We transformed the *scm-2* mutant with the *AtSGP2* promoter::*GUS* construct and we analysed roots from ten T_2 resistant lines. In the *scm-2* mutant background, the pattern of *AtSGP2* promoter activity was disturbed. Stained cells were observed in all cell files, in an identical manner to *GLABRA2* promoter activity in the *scm-2* mutant, but with a weak blue staining (Fig. 3C). This finding suggests that the *SCM* receptor kinase gene is epistatic to *AtSGP2*.

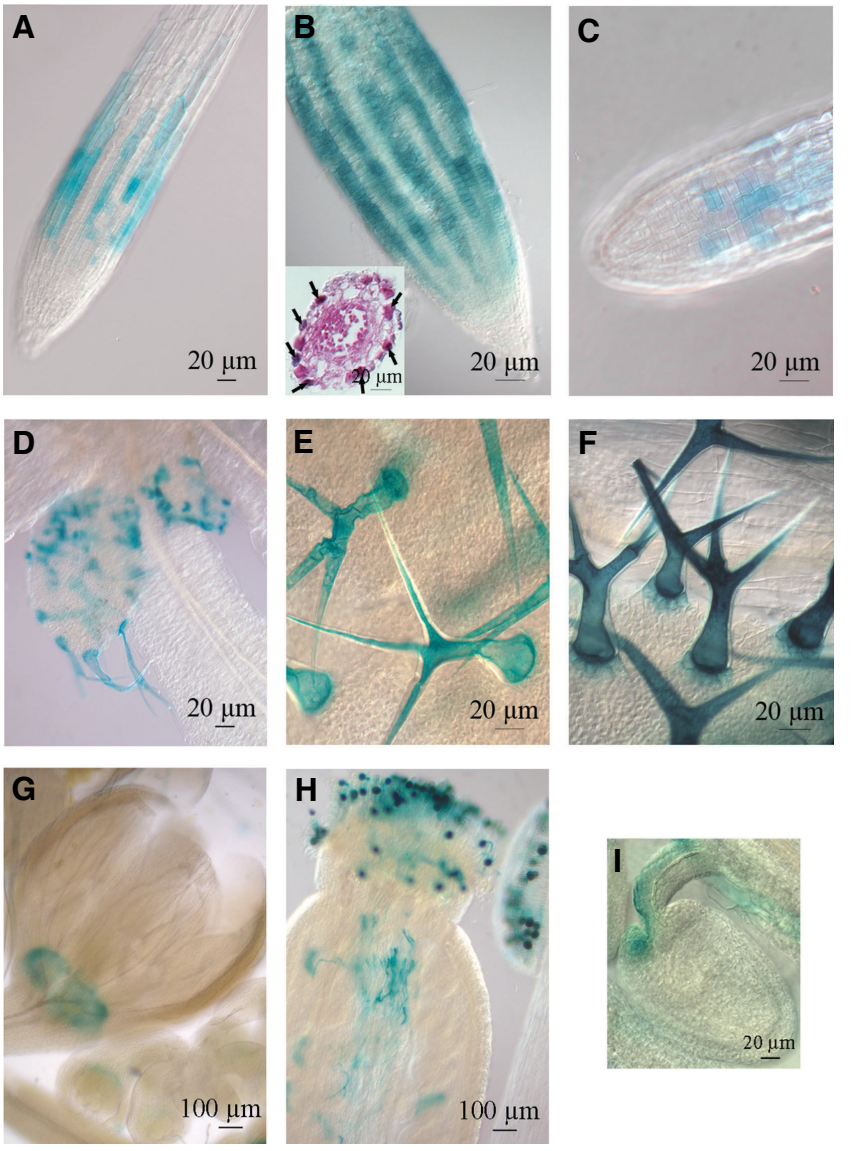

Fig. 3. *AtSGP2* promoter activity in *Arabidopsis thaliana* lines. *Differential GUS staining in the root epidermis in two different 10-day-old T_2 lines* **(A,B)** *and in the* scm-2 *mutant background* **(C)**. *Insert in (B) represents a transverse section in the elongation zone of the primary root: arrows show atrichoblast cells. The* AtSGP2 *promoter is strongly activated during trichome development:* **(D)** *leaf primordium,* **(E)** *young leaf and* **(F)** *mature leaf. No expression is detected in the socket cells. GUS staining is highlighted in the nectaries from young flower buds* **(G)**, *in the pollen tubes at the pollination stage* **(H)** *and during fertilization of the female gametes* **(I)**.

In leaves, expression of the *AtSGP2* promoter was confined to trichomes. During leaf development, GUS activity was found in trichome cells at different developmental stages, from epidermal outgrowth to branch initiation (Fig. 3D) and cell maturation (Fig. 3 E,F). However, the socket cells, arranged around the basal part of the mature trichome, remained unstained (Fig. 3F).

The previously detected high *AtSGP2* transcript levels in flower buds and mature flowers (Champion *et al.,* 2004), prompted us to further explore *AtSGP2* expression in the different flower organs. At early stages of flower development, in flowers from stage 6 / 7 according to Smyth *et al.* (1990), blue staining was found in the receptacle, possibly in nectaries, but not in anthers or ovaries (Fig. 3G). In further stages (stages 10 / 11), blue staining was weak (Fig. 4A). Thereafter in stages 12 / 15, the level gradually increased as pollen matured. *AtSGP2* promoter activity reached a maximum in mature pollen grains at anthesis (Fig. 4A). In order to establish the precise stage of *AtSGP2* promoter activity during male gametogenesis, the nuclei were stained with Hœchst in parallel to the GUS staining. At the uninucleated stage, microspores did not exhibit GUS activity (Fig. 4 B,F,G). After the first asymmetric mitosis (PM I), GUS activity remained undetectable in

most of the young binucleated pollen grains (Fig. 4 C,H,I). At a later stage, a weak *AtSGP2* promoter activity was seen in a few binucleated pollen grains (Fig. 4 D,J,K). After the second symmetric mitosis (PM II), the blue staining of the tricellular pollen showed a significantly increased intensity (Fig. 4 D,L,M), and it peaked in mature pollen at anthesis (Fig. 4 E,N,O).

Given the very strong expression in mature pollen, *AtSGP2* expression was studies after pollination. After anther dehiscence, released blue stained pollen grains germinated on the stigma papillae (Fig. 3H). It was easy to observe the deep blue staining at the distal end of the pollen tubes. Until ovule fertilization, the polarized growth of the pollen tube could be monitored by *AtSGP2* promoter activity. The tips of the pollen tubes penetrate the papillae cuticle and continue to grow intrusively along the stigmatic papillae to the centre of the style into the transmitting tract towards the embryo sacs. *AtSGP2* expression was observed in ovules immediately following fertilization (Fig. 3I).

AtMAP3Kepsilon1 *promoter activity marks some cells targeted by* AtSGP

Interaction between AtSGP1 and BnMAP3Kepsilon1 was previously shown using the yeast two hybrid technique (Champion *et al.,* 2004). A 0.9Kb fragment from the 5' UTR of the *AtMAP3Kepsilon1* gene was cloned upstream of the *GUS* reporter gene. This region corresponds to the promoter region of the *AtMYB5* gene (*At3g13540*), albeit in the reverse orientation. The *AtMAP3Kepsilon1* promoter activity was detected in leaf margins, in trichomes and in root apices of stably transformed *A. thaliana* lines (Fig. 5 A,B). The activity of this promoter was strongly detected in the root cap, both in columella stem cells and columella cells. This expression pattern overlapped with the *AtSGP1* promoter activity in specific root cap cells and therefore the two proteins could interact thus being consistent with the previously observed protein-protein interaction.

A strong *AtMAP3Kepsilon1* promoter activity was also detected in ovules, from early stages until ovule fertilization. In young ovules *AtMAP3Kepsilon1* promoter activity was noticed in the inner integuments (Fig. 5C) that grow to enclose the nucellus. In the mature ovule, *AtMAP3Kepsilon1* promoter activity was detected in the inner and outer integuments, especially in the region surrounding the micropyle (Fig. 5D).

The *AtMAP3Kepsilon1* promoter activity was also examined in leaves where it was observed in trichome cells (Fig. 5B), identical to the *AtSGP2* promoter activity (Fig. 3 D,E,F). This co-expression suggests that *AtSGP2* could potentially interact with *AtMAP3Kepsilon1* in planta, although no such interaction was detected in our yeast two hybrid studies.

Discussion

A fundamental question in biology is how pluripotent cells differentiate towards a specific cell phenotype. This question was addressed through a careful analysis of plant developmental stages using promoter*::GUS* fusions constructs in various genetic backgrounds.

Evolutionary features

A comparison between the chromosome blocks including *AtSGP1* and *AtSGP2* reveals a duplicated segment containing a few genes, with limited synteny. Evidence for an ancient duplica-

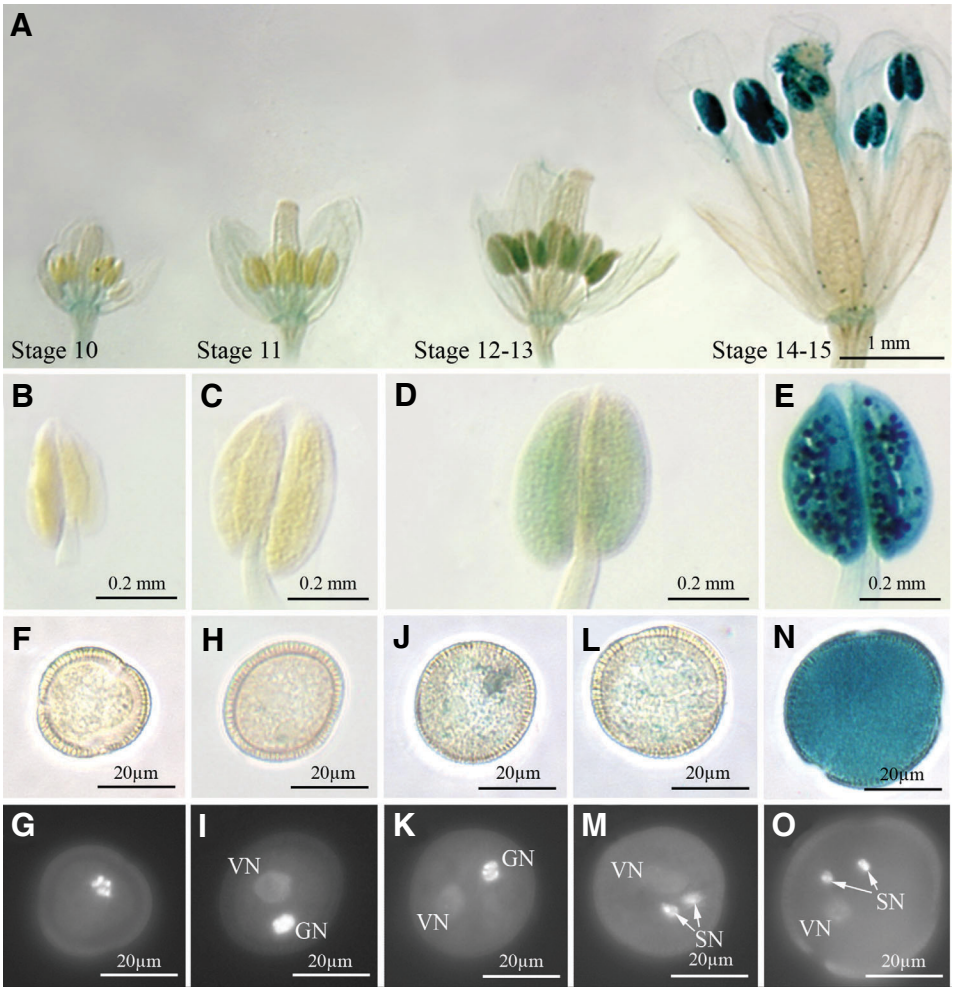

Fig. 4. *AtSGP2* promoter activity during male gametogenesis. *Histochemical localisation of GUS activity in transgenic lines.* **(A)** *Developing and opened flowers from stage 10 to stage 15 according to Smyth (1990).* **(B,C,D,E)** *Magnification of anthers at the different stages are detailed in (A). Microspore to pollen grain isolated from the different stages observed after GUS* **(F,H,J,L,N)** *and Hœchst* **(G,I,K,M,O)** *staining.* **(F,G)** *Microspore.* **(H,I,J,K)** *Bicellular pollen.* **(L,M,N,O)** *Tricellular pollen. Note that a light GUS staining is observed at the late bicellular stage* **(J)**. **(N,O)** *Mature tricellular pollen. Note the intense GUS staining at anthesis. VN: vegetative nucleus; GN: generative nucleus; SN: sperm cell nuclei.*

tion event is mostly provided by the number of synonymous substitutions per site, Ks = 2.83, indicative of a duplication event that pre-dates the Monocot-Eudicot divergence, 125 to 140 million years ago. Both genes and promoters have evolved since that time and the promoters of the paralogues *AtSGP1* and *AtSGP2* now drive activity in different cell types: stomata guard cells, QC, stele and trichomes, atrichoblasts, pollen grains, respectively. Our data provide evidence that these genes putatively play a common role in signalling cell differentiation in different specialized cell types. Our previous work proposed that the two paralogue genes, *AtSGP1* and *AtSGP2* were orthologues of *S. pombe spg1* (Champion *et al.,* 2004). However, their promoter activities reveal that AtSGP proteins do not link cell cycle exit to cytokinesis in plants. This reinforces the recent conclusions of Chaiwongsar *et al.* (2006) who demonstrated that AtMAP3Kepsilon function was not related to that of the core yeast component cdc7p. These proteins have evolved in plants to perform a function different from the SIN pathway (Bedhomme *et al.,* 2008). Evolution seems to have recycled ancient signalling components to derive a new plant signalling pathway in Angiosperms.

AtSGP2 *promoter activity and cell fate specification*

The pattern of *AtSGP2* promoter activity in root epidermal cell files is similar to that of the homeobox transcription factor *GLABRA2* (Masucci *et al.,* 1996). Indeed, cytological observations localised the *AtSGP2* expression in atrichoblasts (non-hair root epidermal cells), in accordance with the gene expression map of the *A. thaliana* root by Birnbaum *et al.* (2003). Root epidermal cells differentiate into two cell types, namely root-hair cells (trichoblasts) and hairless cells (atrichoblasts) in a position-dependent pattern. The trichoblasts lie over the junction of two cortical cells, whereas the atrichoblasts overlie a single cortical cell (Berger *et al.,* 1998a,b; Dolan *et al.,* 1993; Galway *et al.,* 1994). Furthermore, atrichoblast cells exit the mitotic cycle and are directed towards terminal differentiation earlier than their neighbours (Schiefelbein, 2003). For epidermis development, the non functional membrane-bound receptor kinase Scrambled (SCM) (Kwak *et al.,* 2005) enables epidermal cells to perceive positional cues (Llompart *et al.,* 2003). The *SCM* gene is required for proper position-dependent cell-type patterning. We observed that the *AtSGP2* promoter activity pattern in the root epidermis of the *scm-2* mutant was identical to the pattern of *GLABRA2* in the *scm-2* mutant. This led us to postulate that *SCM* is epistatic to *AtSGP2*, both participating in a putative signalling pathway that acts early to promote position-dependent cell fate specification of non hair cells.

A similar set of proteins controls the patterning mechanism of hairs in the leaf epidermis (trichomes), of non hair cells in the root epidermis (atrichoblasts), of stomata in the hypocotyl epidermis (not detailed here) and of seed coat cells (pigments and mucilage) (Pesch and Hulskamp, 2004; Schiefelbein, 2003; Zhang *et al.,* 2003). *AtSGP2* promoter activity suggests its involvement in trichome cell fate specification. Similarly the *AtMAP3Kepsilon1* promoter shows activity in trichome cells. This co-expression with *AtSGP2* suggests that both proteins could interact in planta. It is interesting to note that *AtSGP1* promoter activity is detected in stomata guard cells, whereas *AtSGP2* and *AtMAP3Kepsilon1* promoter activities were observed in trichomes. Results from Glover *et al.* (1998) indicated that in the leaf epidermis, the cell differentiation programs for stomata and trichomes arise from the same pool of uncommitted cells.

Furthermore, *AtMYB5* promoter-driven GUS activity was previously detected by Li *et al.* (1996) in leaf trichomes and in the seed coat of the young seed. These observations are identical to those reported in this work for the *AtMAP3Kepsilon1* promoter (which consists of the same genome region but in the reverse orientation). This provides evidence that both genes are involved in trichome and seed coat cell fate specification.

AtSGPs *promoter activity and maintenance of cell differentiation*

GFP driven by the synthetic *DR5* promoter co-localized with *AtSGP1* promoter activity, suggesting that *AtSGP1* is mainly expressed in the QC. This conclusion agrees with the transcriptome analyses of Birnbaum *et al.* (2003) and Brady *et al.* (2007). The QC is an organizing centre which maintains the stem cell function of initials (van den Berg *et al.,* 1997). In contrast, cells from the QC display a reduced mitotic activity. *PLT1* and *PLT2* genes are redundantly required for the specification of organizing QC cells and for the maintenance of root stem cells. In *plt1 plt2* double

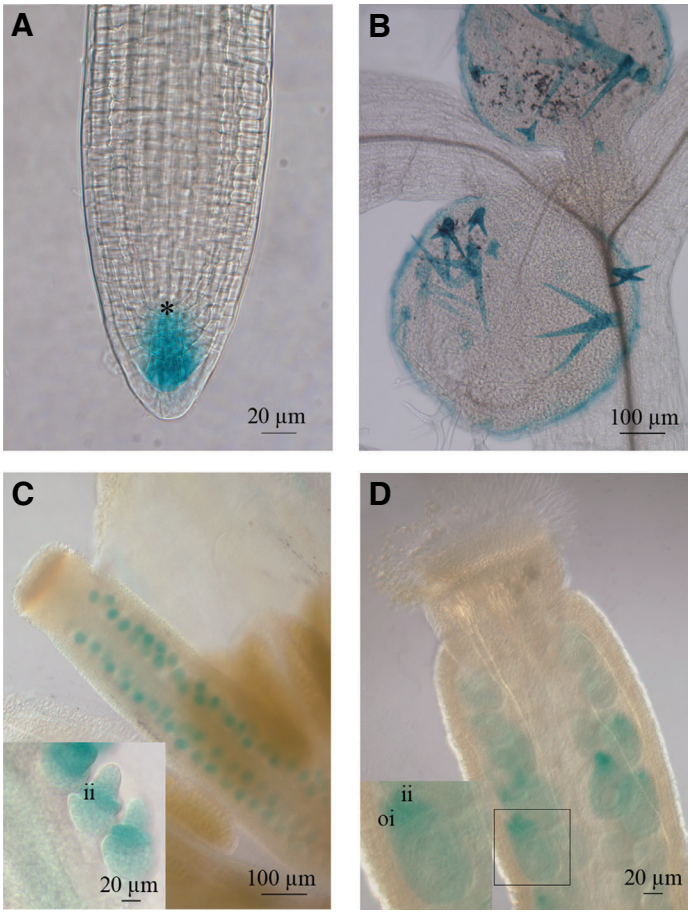

Fig. 5. *Arabidopsis thaliana AtMAP3Kepsilon1* promoter activity in transgenic T_2 lines. *Collumella cells at the root tip* **(A)**. *QC is marked by an asterisk. Trichomes from the young leaf epidermis* **(B)**. **(C)** *Carpels with young developing ovules. Insert in (C) represents a detailed view of the same ovule stage; ii: inner integument.* **(D)** *In mature ovules, the GUS staining is observed in the micropyle region as shown in the insert; ii: inner integument and oi: outer integument.*

mutants, QC and stem cell identity are lost (Aida *et al.,* 2004) and *AtSGP1* promoter activity was seen to spread throughout additional layers of differentiated cells, proximal to the mis-specified QC cells. Therefore a functional QC was not necessary for *AtSGP1* promoter activity. This suggests that *PLT* does not genetically interact with *AtSGP1*. Nevertheless, *PLT* expression is required to restrict *AtSGP1* promoter activity to the QC, meaning that, genetically, *PLT* is upstream of *AtSGP1*. The wide distribution of *AtSGP1* in the root cap of *plt1 plt2* double mutants confirms that *AtSGP1* also specifies the differentiated collumella cells. Interestingly, our results show an overlap between the promoter activity profiles of *AtSGP1* and *AtMAP3Kepsilon1* in the columella cells. This co-expression indicates that they could be protein partners in planta. Their physical interaction was previously detected in yeast double hybrid studies (Champion *et al.,* 2004).

We also found *AtSGP1* promoter activity in the stele, particularly at stages that include differentiated cells. Again, such observations agree with the recent root transcriptome map of Brady *et al.* (2007). Our data set suggests that *AtSGP1* could also be involved in specification and maintenance of stele cell differentiation.

The asymmetric division of a leaf epidermal cell produces a primary meristemoid and a pavement cell. The meristemoid undergoes one, two or three asymmetric mitosis before guard mother cell specification (Donnelly *et al.,* 1999). The last mitosis of the guard mother cell is symmetric and produces the two guard cells (Larkin *et al.,* 1997). *AtSGP1* promoter activity was only detected in mature stomata guard cells. *AtSGP1* promoter activity in these cells suggests that *AtSGP1* has a role in determining the function of these differentiated cells.

Finally, we noticed that the *AtSGP2* promoter activity was very strong in mature pollen. In *A. thaliana*, asymmetric division of a uninucleated microspore in pollen mitosis I (PMI) produces a large vegetative cell and a small generative cell, each having a different fate. Whereas the vegetative cell does not further divide, the generative cell divides symmetrically to produce two sperm cells. The *AtSGP2* promoter become active after the asymmetric division of the male gametophyte, and it continues to be active during pollen tube growth into the embryo sac. This suggests that *AtSGP2* promoter activity is required for the determination of pollen function. The *AtSGP2* promoter activity in male gametophytes observed in this work is in full accordance with the transcriptome analyses from Pina *et al.* (2005) and Honys and Twell (2004). About 500 *A. thaliana* genes exhibit a pollen expression profile identical to *AtSGP2*, most of them with a reduced level. The promoter activity during pollen tube growth should be very useful to check for pollination, fertilisation and self-incompatibility response.

Moreover, *AtSGP* genes could play a role either in early mechanisms in cell fate determination (atrichoblasts, integuments, trichomes) or later in the determination of cell function (QC, stomata guard cells, pollen, stele). This suggests that *AtSGP1* and *AtSGP2* promoters can be used as cellular markers of early and late events in cell differentiation. Our data should be used as a starting point towards genetic and functional analyses. We are presently designing experiments to test the hypothesis that the two paralogous GTPase genes, *AtSGP1* and *AtSGP2,* are signalling components involved in mechanisms of cell differentiation.

Studies in Mammalian systems have revealed that pluripotency is controlled by a regulatory core of transcription factors that activate genes critical for self renewal of stem cells and repress genes initiating differentiation (Sun *et al.,* 2006; Wang *et al.,* 2006). Similar data from plant cells and mammalian cells suggest that stem cells can be specified by kingdom specific patterning mechanisms, connected to related epigenetic stem cell factors (Ben Scheres, 2007). What component combinations signal and confer differentiation to plant specific cell types remain to be elucidated.

Materials & Methods

Plant material and transformation

Constructions of *AtSGP1* and *AtSGP2* promoter-GUS gene fusions were introduced into *Agrobacterium tumefaciens* (HBA105 and Agl1 respectively). *Arabidopsis thaliana* plants (Columbia ecotype or mutant lines) were transformed by the floral dip method as described (Clough and Bent, 1998). The progeny of the T_0 plants was sown on a 0.5X Murashige and Skoog (MS) medium, complemented with kanamycine (50 mg/mL). After 2 weeks, a fraction of the resistant T_1 plantlets were tested for GUS staining while the remaining plants were transferred to soil in a greenhouse under short-day conditions for 2 weeks, then under long-day conditions. The T_1 and T_2 generations were selected on kanamycin.

AtSGP1, AtSGP2 *and* AtMAP3Kepsilon1 *promoter::GUS fusion constructs*

Promoter regions were amplified from *Arabidopsis thaliana* genomic DNA (Columbia ecotype) by PCR. Using primers containing added restriction sites, a 2.2 kb region upstream from the ATG start codon was chosen for *AtSGP1* (*At5g54840*) and a 1.2 kb region for *AtSGP2* (*At3g21700*). The *AtSGP1* primers were:
G1proEcoPst 5'-CGGAATTCTGCAGGGGCGGATACATGAATAAAC, and G1proBam 5'-CGGGATCCTGATGAAACAGAGAGAAAGTTATGTG. The *AtSGP2* primers were:
G2proEcoR1 5'-CAGAATTCGCCAATGATGGTGAAAGATGTAG, and G2proSal1 5'-ACGTCGACTGAGAAAATTCAAAATTCGAAATC. The *AtSGP1* promoter was introduced into the pTAK plasmid, upstream of the β-glucuronidase (GUS) gene as a Pst1/BamH1 fragment. The *AtSGP2* promoter PCR fragment was ligated into pGEM-T (Promega, Madison, WI). The DNA sequence of both cloned promoter region sequences was verified before subsequent cloning. For the *AtSGP1* promoter, a 1.2 kb promoter region plus the GUS gene was excised from the construct in pTAK as a HindIII/EcoRI fragment and subcloned into the pCW83 binary plasmid. The promoter fragment of *AtSGP2* obtained by digestion (EcoRI/SalI) was transferred into the pPR97 binary vector upstream of the GUS gene. As a result for both *AtSGP1* and *AtSGP2*, a 1.2 kb promoter region was used for subsequent promoter activity analyses. For *AtMAP3Kepsilon1* (*At3g13530*) a 0.9 kb region upstream of the ATG start codon was chosen. The *AtMAP3Kepsilon1* gene is localised upstream of the *AtMYB5* gene in the reverse orientation (Jouannic *et al.,* 2001). This means that the *AtMAP3Kepsilon1* promoter region is also the promoter of the *At3g13540* (*MYB5*) gene, and this has been previously cloned (Li *et al.,* 1996). The primers used for PCR amplification were: 3Ke1proEcoR1 5'-CAGAATTCTCTTTCCACTAGGGTTTCGTG, and 3Ke1proSal1 5'-ACGTCGAC CTCTTCTCGTTTCTTCTTCCTCC. The promoter fragment of *AtMAP3Kepsilon1* was cloned as described for the *AtSGP2* promoter. The final constructs were transferred into *Agrobacterium* by electroporation.

Histochemical GUS and Hœchst staining

Histochemical localization of GUS activity was performed on 10- or 12-day-old T_1 and T_2 generation seedlings and on inflorescences from T_1 and T_2 plants at various stages of floral development (unopened bud to mature

silique). Samples (from at least 10 different lines) were fixed in cold 90 % acetone for 30 min. They were washed with staining buffer (0.5 mM sodium phosphate buffer, 5 mM ferrocyanide, 5 mM ferricyanide) and placed in X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) staining solution (staining buffer containing 39 mM X-Gluc from a 767 mM stock solution in DMSO). Tissues were vacuum-infiltrated for 10 min and incubated at 37 °C for 1.5 h. Finally the GUS buffer was replaced by 30 % ethanol during 20 mins, 50 % ethanol during 20 min and 70 % ethanol for 20 min, then left overnight to destain the tissues prior to observation. Before microscope observation, ethanol was removed and samples were cleared by an overnight incubation in chloral hydrate solution (chloral hydrate / glycerol / water 8:2:1(w/v/v)).

For pollen nuclei visualization, samples were fixed in ethanol:acetic acid (3:1) for 10 min just after flower GUS staining and rehydrated in and ethanol series (70 %, 50 %, 30 %) and finally water. Pollen walls were digested by incubation in a solution containing 0.3 % pectolyase, 0.3 % cytolyase and 0.3 % cellulose for 30 min at 37 °C. Enzymes were eliminated by 3 washes in water and DNA was stained with 5 μg/mL Hœchst in 1 % Triton X100 and visualised under UV light.

For sectioning, GUS-stained root segments were fixed in 3 % glutaraldehyde diluted in PBS 1X (pH 7.2) and washed 4 times in 1X PBS (pH 7.2) for 10min. Then samples were dehydrated successively in ethanol (,30 %; 50 %, 70 %, 80 %, 95 % and 100 %) with a 15 min incubation at each concentration. The plantlets were soaked in 1/3 histoclear 2/3 ethanol 100 % for 15 min, 1/2 histoclear 1/2 ethanol 100 % with eosine for 15 min, histoclear pure for 15 min and overnight in 1/2 histoclear (60 °C) 1/2 ParaplastPlus® (60 °C). The next day, roots were embedded in Paraplast Plus® and cut into 8 μm-thick sections using a microtome.

Starch granules were visualised in the collumella as described by Willemsen *et al.* (1998).

Light and confocal microscopy

Images of *Arabidopsis thaliana* roots and inflorescences stained in X-Gluc solution were captured on a Zeiss Axioskop Imaging microscope equipped with a Sony Power HAD camera (Paris, France) and the Axiovision 1.01 (Zeiss, Jena, Germany) software, and processed using Adobe Photoshop 6 software.

Roots of the DR5::GFP line were observed using a confocal microscope (Leica SP2). For GFP detection, excitation was performed at 488 nm, with fluorescence emission captured between 510 and 514 nm.

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