Int. J. Dev. Biol. 55: 657-664
doi: 10.1387/ijdb.113340er

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Reproductive Meristem22 is a unique marker for the early stages of stamen development

ELISSON ROMANEL[1], PRADEEP DAS[2], RICHARD M. AMASINO[3], JAN TRAAS[2], ELLIOT MEYEROWITZ[4] and MARCIO ALVES-FERREIRA*,[1]

[1]Laboratório de Genética Molecular Vegetal (LGMV), Departamento de Genética, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil, [2]Laboratoire RDP, Ecole Normale Supérieure de Lyon (ENS), Université de Lyon, Lyon, France, [3]Department of Biochemistry, University of Wisconsin, Madison, WI, USA and [4]Division of Biology, 156-29, California Institute of Technology, Pasadena, California, USA

ABSTRACT Stamens undergo a very elaborate development program that gives rise not only to many specific tissue types, but also to the male gametes. The specification of stamen identity is coordinated by a group of homeotic genes such as *APETALA3 (AP3)* and *PISTILLATA (PI)*, *AGAMOUS (AG)* and *SEPALLATA (SEP1-4)* genes. Genome-wide transcriptomic comparisons between floral buds of wild-type and *ap3* mutants led to the identification of the *REM22* gene, which is expressed in the early stages of stamen development. This gene is member of the plant-specific B3 DNA-binding superfamily. In this work, we dissect the spatio-temporal expression pattern of *REM22* during the early stages of stamen development. To this end, both *in situ* hybridization analyses as well as *in vivo* fluorescence strategies were employed. At stage 4 of flower development, *REM22* is expressed exclusively in those undifferentiated cells of the floral meristem that will give rise to the stamen primordia. At stage 5, *REM22* expression is restricted to the epidermal and the subepidermal layers of anther primordia. Later, this expression is confined to the middle layer and the differentiating tapetal cells. After stage 10 when all the tissues of the anther have differentiated, *REM22* expression is no longer detectable. Furthermore, we examined the *pREM22::GUS-GFP* marker line in an inducible system where the ectopic AG function is used to promote microsporogenesis. The data support the idea that *REM22* expression is a useful marker to study the early stages of stamen development.

KEY WORDS: *male sporogenesis, B3 domain, reproductive meristem, stamen development, REM22 function*

The development of the *Arabidopsis* flower is divided into 12 stages (Smyth *et al.*, 1990). Medial anther primordia are visible at stage 5 of flower development and this corresponds to stage 1 of stamen development (Sanders *et al.*, 1999). Stamen development is divided into 14 stages and it ends when the stamen falls off in the senescing flower. Cell division and tissue differentiation occur from stages 1 to 7 of stamen development and these processes culminate with the end of meiosis and the formation of tetrads of free microspores within each locule. At stage 1 of stamen development, cell divisions in the epidermal layer 1 (L1), the sub-epidermal L2 and inner L3 layers of the floral meristem result in the formation of stamen primordia. During stage 2, the cells in the L2 layer divide periclinally (perpendicularly to the layer) to give rise to archesporial cells (AC). At stage 3, the archesporial cells themselves divide periclinally to give rise to the primary parietal (PP) and the primary sporogenous (PS) layers. At stage 4, the PP layer cells divide periclinally to give rise to the inner secondary parietal layer (ISP) and the outer secondary parietal layer (OSP). At stage 5, OSP cells divisions result in the formation of the endothecium, ISP cells divide periclinally to form a middle layer and tapetal cell and, lastly, PS cells divide to form microspore mother cells.

The patterning of floral organ identity is controlled by the combinatorial activities of the floral homeotic genes, as represented in the ABCE model (Coen and Meyerowitz, 1991, Ditta *et al.*, 2004, Goto *et al.*, 2001, Melzer and Theissen, 2009, Melzer *et al.*, 2009,

Abbreviations used in this paper: AG, AGAMOUS; AP3, APETALA3; DEX, dexamethasone; PI, PISTILLATA; REM, reproductive meristem; SEP, SEPALLATA.

***Address correspondence to:** Márcio Alves-Ferreira. Laboratório de Genética Molecular Vegetal (LGMV), Departamento de Genética, Universidade Federal do Rio de Janeiro (UFRJ), Av. Prof. Rodolpho Paulo Rocco, CCS 21949900 Rio de Janeiro, Brazil. Tel: + 55-21-2590-0111. Fax: +55-21-2590-0111. e-mail: marcioaf@ufrj.br

Supplementary Material (a video, two figures and a table) for this paper is available at: http://dx.doi.org/10.1387/ ijdb.113340er

Accepted: 27 July 2011. *Final, author-corrected PDF published online:* 24 August 2011. *Edited by:* Mieke van Lijsebettens.

ISSN: Online 1696-3547, Print 0214-6282
© 2011 UBC Press
Printed in Spain

Pelaz *et al.*, 2000). Stamen identity requires the so-called B, C and E function genes, *APETALA3* (*AP3*), *PISTILLATA* (*PI*), *AGAMOUS* (*AG*) and *SEPALLATA 1-4* (*SEP1-4*). In the last few years, several genes involved in early stages of stamen development have been identified, such as *NZZ*/*SPL* (*NOZZLE*/*SPOROCYTELESS*) (Schiefthaler *et al.*, 1999, Yang *et al.*, 1999); *EXS*/*EMS1* (*EXTRA SPOROGENOUS CELLS*/*EXCESS MICROSPOROCYTES1*) (Canales *et al.*, 2002); *TPD1* (*TAPETUM DETERMINANT1*) (Yang *et al.*, 2003); *SERK1/2* (*SOMATIC EMBRYOGENESIS1/2*) (Albrecht *et al.*, 2005); *BAM1/2* (*BARELY ANY MERISTEM1/2*) (Hord *et al.*, 2006); *DYT1* (*DYSFUNCTIONAL TAPETUM1*) (Zhang *et al.*, 2006); *RECEPTOR-LIKE PROTEINS KINASE2* (*RPK2*) (Mizuno *et al.*, 2007); and *ROXY1/2* (Xing and Zachgo, 2008).

Although several mutants with defects at the earliest stages of stamen development have been identified, the molecular events necessary for stamen formation and microspore development are still poorly understood. To fill this gap, several genome-wide analyses have recently been performed in *Arabidopsis thaliana* to identify the target genes of the ABCE complexes (Alves-Ferreira *et al.*, 2007, Gomez-Mena *et al.*, 2005, Ito *et al.*, 2004, Kaufmann *et al.*, 2009, Wellmer *et al.*, 2006). These studies have revealed the expression of several members of the plant-specific B3 superfamily during the early stages of flower, stamen and carpel development. B3 superfamily genes encompass five families, namely ABI3 (ABSCISIC ACID INSENSITIVE3) (Suzuki *et al.*, 1997), HSI/VAL (High-level expression of Sugar-Inducible gene/VP1/ABI3-like) (Suzuki *et al.*, 2007, Tsukagoshi *et al.*, 2005), ARF (Auxin Response Factor) (Ulmasov *et al.*, 1997), RAV (Related ABI3/VIVIPAROUS) (Kagaya *et al.*, 1999) and REM (Reproductive Meristem) (Franco-Zorrilla *et al.*, 2002). Most of the B3 genes thus identified in reproductive organs belong to the REM family (Romanel *et al.*, 2009). The REM family is composed by 45 members in *Arabidopsis*, although only two were recently characterized, *VERNALIZATION1* (*VRN1*) and *VERDANDI* (*VDD*) (Levy *et al.*, 2002, Matias-Hernandez *et al.*, 2010). *In situ* expression analyses of several of these REM family genes have shown distinctive spatial expression patterns at specific stages of flower, stamen or carpel development (Alves-Ferreira *et al.*, 2007, Gomez-Mena *et al.*, 2005, Matias-Hernandez *et al.*, 2010, Wellmer *et al.*, 2006, Wijeratne *et al.*, 2007).

REM22 expression was observed at the earlier stages of stamen and carpel development in previous works (Alves-Ferreira *et al.*, 2007, Gomez-Mena *et al.*, 2005). However the expression pattern was not carefully described and only limited data are available about *REM22* tissue specific expression. To test the value of *REM22* as a marker of stamen development, we performed a detailed *in situ* hybridization analysis during stamen development. In parallel, we also fused the promoter region of *REM22* to the GUS/GFP reporter genes and evaluated the transgenic lines by *in vivo* confocal microscopy. This construct was also evaluated in mutants defective in stamen development. Our results provide direct evidence that *REM22* expression is a distinctive marker for flower meristem cells undergoing differentiation into stamen primordia. At later stages of flower development, *REM22* is expressed strongly and exclusively in the L2-derived cells undergoing differentiation, but not in microspore mother cells and their descendents. This expression pattern is unique and may be valuable to study stamen cell differentiation. The expression of the *pREM22::GUS-GFP* marker line is very similar to the observed *in situ* hybridization data, which makes this line a valuable tool for the study of stamen formation and anther development.

Results

REM22 *is expressed during the differentiation of parietal cells in the early stages of stamen development*

To gain a more detailed spatial-temporal understanding of *REM22* expression during flower development, we performed a comprehensive *in situ* hybridization analysis (Fig. 1). *REM22* transcripts were detected at stage 3 of flower development in L1 and L2 cell layers (Fig. 1A). At stage 5 of flower development, the transcripts were also detected in the L1 and L2 layers that later generate epidermis and archesporial cells, respectively (Fig. 1B). At stage 6, high *REM22* expression is detected in archesporial cells, but the signal is weak in the epidermis (Fig. 1C). *REM22* expression was also detected in parietal cells and in the inner secondary parietal layer. At stage 8 of flower development, *REM22* expression is no longer visible in the epidermis and is weakly detected in the outer secondary parietal layer. *REM22* is also expressed during carpel development (Fig. 1 D,E,F). At stages 8 and 9 of flower development, a stronger expression was observed in the L2 than in the L1

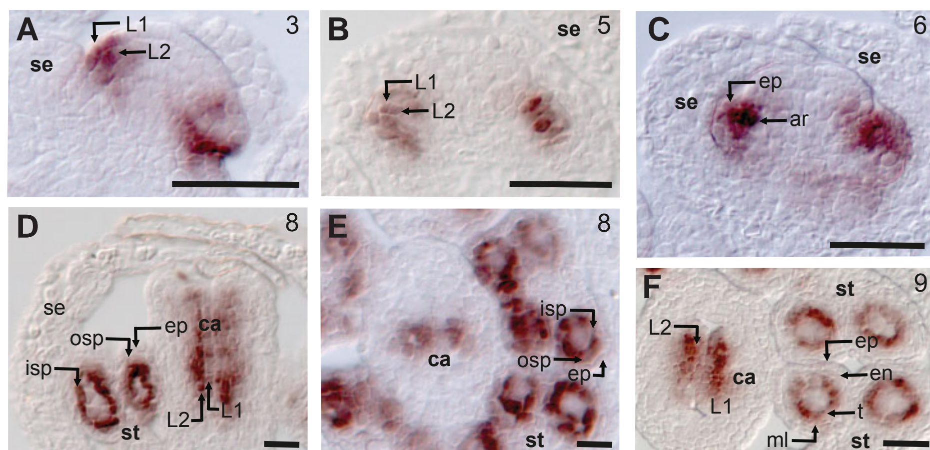

Fig. 1. Expression pattern of *REM22* during early stages of wild-type *Arabidopsis* flower development. (A) *Stage 3 flower showing expression of REM22 in L1 and L2 cells.* **(B)** *Stage 5 flower showing REM22 expression in L1 and L2 cells.* **(C)** *Stage 6 flower showing strong* REM22 *expression in the archesporial cells and a weaker signal in the epidermis.* **(D)** *Stage 8 flower bud longitudinal section, showing higher expression of* REM22 *in ISP than OSP.* REM22 *is also expressed in L1 and L2 of the carpel primordia (arrows).* **(E)** *Stage 8 flower bud cross section showing* REM22 *expression in carpels and stamens.* **(F)** *Stage 9 flower buds,* REM22 *shows a high expression in tapetal cells, but it is expressed also in the middle layer cells. Numbers at the upper-right corner of the figures indicate approximate flower stage. L1, layer 1; L2, layer 2; ca, carpel; se, sepal; st, stamen; ar, archesporial cells; ep, epidermis; osp, outer secondary parietal cell; isp, inner secondary parietal cell; en, endothecium; ml; middle layer; t, tapetum. Scale bar: 25μm. Arrows indicate regions of expression in specific tissues of stamen and carpel.*

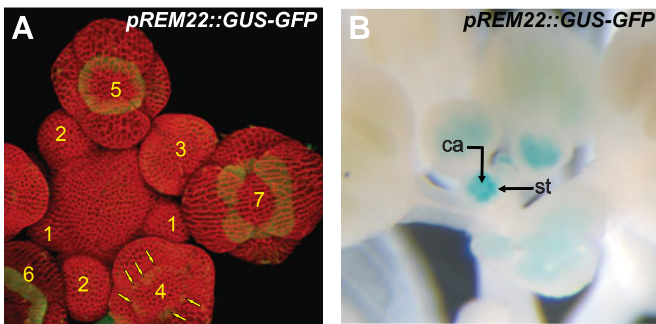

Fig. 2. Confocal microscopy analysis of *REM22* promoter activity in wild-type plants. (A) *wild-type inflorescence projection of* pREM22::GUS-GFP *showing weak GFP signal at stage 4 flower bud (arrows) and strong GFP signal at later stages (stage 5 to 7) of flower development. Cell membranes were marked with the dye FM4-64 and GFP fluorescence can be seen as a green signal.* **(B)** *wild-type inflorescence of* pREM22::GUS-GFP *showing GUS staining in stamens (arrows). ca, carpel; st, stamen.*

of carpel primordia. The ovule primordia arise in the L2 layer of carpel primordia in stage 9 of flower development. *REM22* expression in male and female reproductive development is similar if we take into consideration tissue differentiation during stamen and carpel development, suggesting that this gene may have a similar function in both processes. At stage 9 of flower development, *REM22* hybridization signal was strong in tapetal cells and low in the middle cell layer (Fig. 1 D,E,F). Finally, at late flower development stage 9, when the stamen tissues are fully differentiated, no signal is observed in any stamen tissue (data not shown).

The expression pattern of *REM22* suggests that it may have an important function during *Arabidopsis* reproductive development. To check this possibility, a T-DNA insertion line for the gene At3g17010 (*REM22*) was characterized. In the homozygous insertion line for At3g17010 (in which the T-DNA lies in the second exon of the gene), the corresponding complete gene transcripts were not detectable (Supplementary Fig. 1). The T-DNA insertion line had no discernable mutant phenotype (data not shown).

REM22 *reporter lines display expression similar to* in situ *hybridization expression patterns*

In order to obtain a marker line for the *REM22* expression pattern, we cloned a fragment with 1022 bp upstream of the ATG start codon. This DNA fragment corresponds to the entire genomic region between *At3g17010* and the upstream gene *At3g17000* (Supplementary Fig. 2). The fragment was fused to the reporter genes GUS/GFP in the vector pKGWFS7 by recombination and the resultant construct, named *pREM22::GUS-GFP*, was transformed into *Arabidopsis thaliana* wild-type plants using the floral dipping method (Clough and Bent, 1998). Five out of fifteen primary trans-

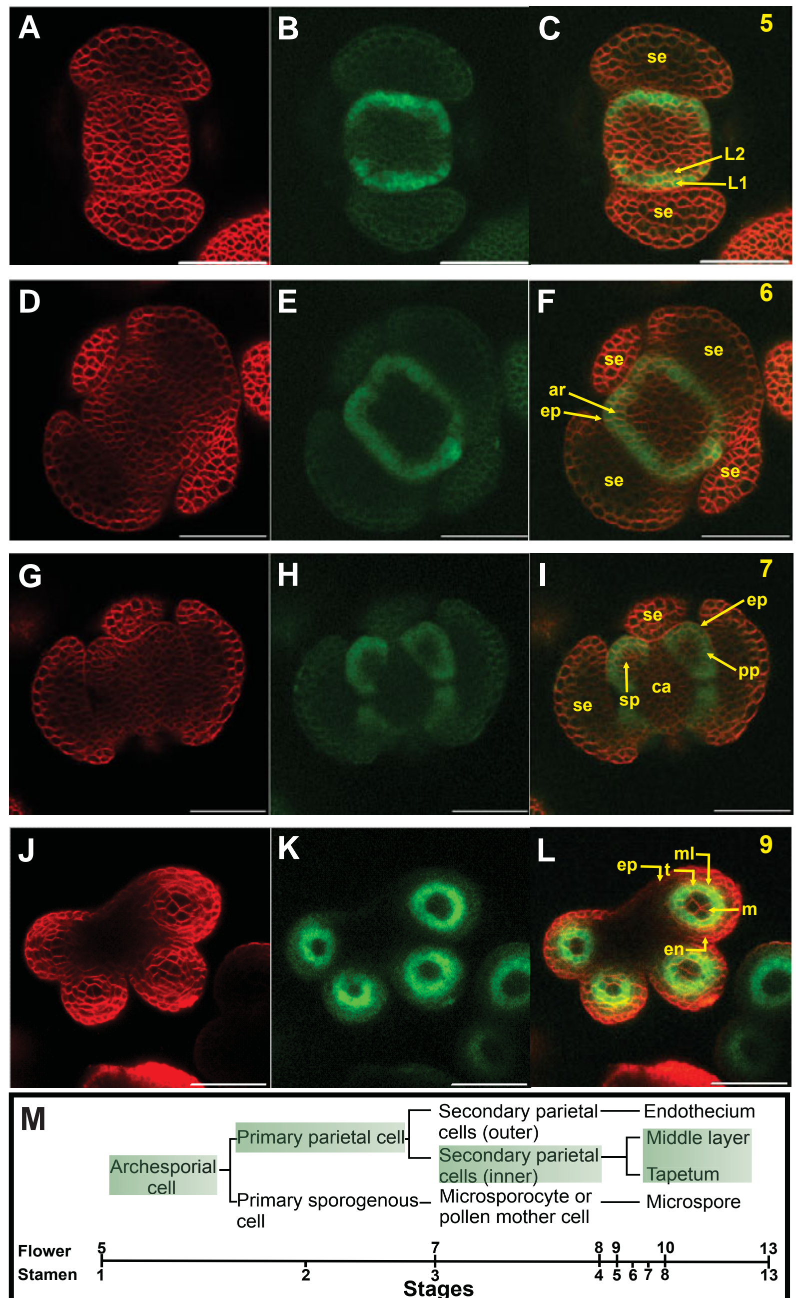

Fig. 3. Confocal microscopy of REM22 promoter activity in *pREM22::GUS-GFP* plants at early stages of stamen development. (B,E,H,I) *GFP fluorescence can be seen as a bright green signal.* **(A,D,GJ)** *Cell membranes marked with the dye FM4-64, shown in red.* **(C,F,I,L)** *Merged confocal images from A/B, D/E, G/H and J/K figures.* **(C)** *Predominant localization of GFP signal in L1 and L2 cells in the third whorl of a stage 5 flower bud.* **(F)** *GFP signal in epidermis and archesporial cells at a stage 6 flower bud.* **(I)** *GFP signal in epidermis and primary parietal cell at stage 7 flower bud.* **(L)** *stage 9 flower buds shows a strong GFP signal in the tapetum layer and a weak signal in the middle layer.* **(M)** *Diagram depicting cell lineages during wild-type anther development. The green boxes enclose cell types were the expression of* REM22 *is observed. The bar below the diagram shows stages of flower and anther development described by Smyth* et al., *(1991) and Sanders* et al., *(1999), respectively (adapted from Alves-Ferreira* et al.; *2007). Numbers at the upper-right corner of the figures indicate approximate flower stage. L1, layer 1; L2, layer 2; ca, carpel; se, sepal; st, stamen; ar, archesporial cells; ep, epidermis; pp, primary parietal cell; sp, sporogenous primary; en, endothecium; ml; middle layer; t, tapetum; m, microspore. Scale bar: approximately 50 μm.*

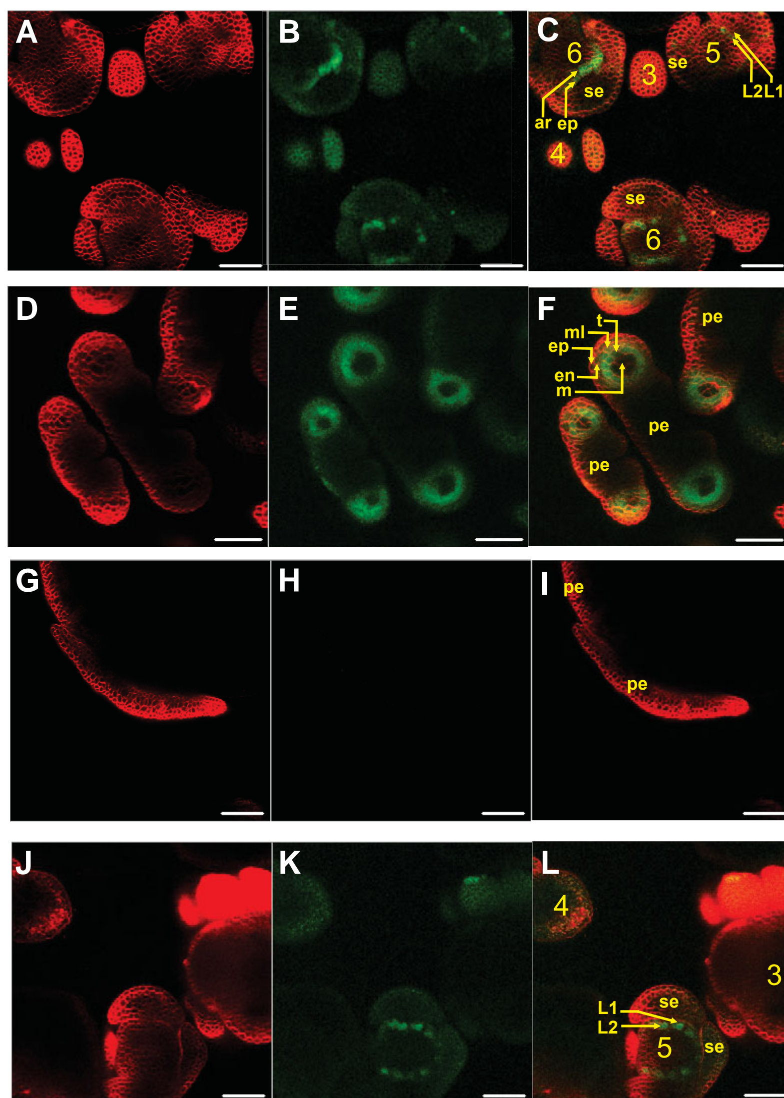

Fig. 4. Confocal microscopy of *pREM22::GUS-GFP* promoter activity in *p35S::AG-GR, ag* background. *GFP fluorescence can be seen as a bright green signal in* **(B,E,K)**. **(A,D,G,J)** *Cell membranes marked with the dye FM4-64 are shown in red.* **(C,F, I,L)** *Merged confocal images of A/B, D/E, G/H and J/K.* **(C)** *Inflorescence of* p35S::AG-GR, ag/ pREM22::GUS-GFP *line under continuous DEX treatment showing GFP signal at stage 5 and 6 flower bud.* **(F)** p35S::AG-GR, ag *treated with DEX at a late stage of flower development showing GFP signal in staminoid petals in different stages of development.* **(I)** p35S::AG-GR, ag/ pREM22::GUS-GFP *not treated with DEX at late stage showing no change in petals and no GFP expression.* **(L)** *Inflorescence of* p35S::AG-GR, ag/ pREM22::GUS-GFP *showing GFP signal at stages 5 flower bud without the presence of functional AG protein. Numbers in* **(C,L)** *indicate the flower stage. L1, layer 1; L2, layer 2; se, sepal; pe, petal; ep, epidermis; en, endothecium; ml, middle layer; t, tapetum; m, microspore. Scale bar: 50 μm.*

formants (#17.02; #17.08; #17.11; #17.13 and #17.15) showed a segregation of 3:1 for antibiotic resistance, indicating the presence of only one T-DNA locus in these lines. Homozygous transformant lines were obtained and the GUS expression was evaluated in reproductive tissues during development. Three out of the four homozygous lines (#17.02; #17.08 and #17.13) showed GUS staining during anther and ovule development (Fig. 2B) similar to the expression pattern observed previously by *in situ*.

To better investigate the spatial and temporal expression pattern of *REM22*, we used confocal-based live imaging in the line *pREM22::GUS-GFP*/#17.13 during the early stages of flower development. GFP expression was observed at stages 4 to 9 of flower development (Figs. 2A and 3). While only a very low expression is detected in L1 and L2 cells during stage 4 of flower buds in the line #17.13 (Fig. 2A, Supplementary Video 1), our *in situ* results clearly show that *REM22* expression in L1 and L2 cells begins at stage 3 (Fig. 1A). The absence of GFP signal at stage 3 of flower development and weak GFP signal at stage 4 could be explained by the sensitivity of techniques and/or absence of some *cis* elements in the promoter fragment we used in the construct.

At stage 5 of flower development, when the petals and stamen whorls are well defined, the GFP signal is observed exclusively in L1 and L2 cells of the third whorl, in agreement with the *in situ* hybridization analysis (Fig. 3 A-C). At stage 6 of flower development, GFP expression appears to become restricted to the L1 and L2 cells that have begun to differentiate into archesporial cells (Fig. 3 D-F). This expression pattern is similar to *REM22* transcripts, though the *in situ* hybridization data showed a stronger hybridization signal in the archesporial cells when compared to the epidermis. At stage 7 of flower development, GFP expression continues in the epidermis and primary parietal layer (Fig. 3 G-I). We also can observe GFP expression in cells of the inner secondary parietal layer. At this stage, however, no *REM22* RNA is detected in the epidermis. One explanation for these minor discrepancies could be that the GFP-GUS chimeric protein is more stable than REM22 transcripts, and it persists through cell divisions.

At the stage 9 of flower development, when all tissues of mature stamens are present, GFP signal is observed exclusively in tapetal cells and in the middle cell layer (Fig. 3 J-L). These data overlap perfectly with *REM22 in situ* hybridization data at stages 8 and 9. *REM22* expression is weaker in the outer secondary parietal cells than in the inner secondary parietal cells (ISPC), which differentiate into the middle cell layer and tapetal cells (Fig. 3M). The expression of *REM22* observed by *in situ* as well as *in vivo* experiments showed that *REM22* is expressed at high level in ISPC until early stage 10 of flower development, when these tissues are not totally differentiated.

Spatial and temporal expression patterns of pREM22::GUS-GFP *in the inducible AGAMOUS function system*

To determine how useful the *pREM22::GUS-GFP* reporter line could be in studying stamen development, we crossed the line with *p35S::AG-GR, ag* background that is an excellent tool to examine male reproductive development.

We exploited an inducible system where an ectopic AG function is used to promote microsporogenesis. This *p35S::AG-GR, ag* line constitutively expresses an inducible version of AG fused to the rat glucocorticoid domain GR (Ito *et al.*, 2004). The chimeric AG-GR protein is inactive in the cytoplasm, but after exposure to the synthetic glucocorticoid dexamethasone (DEX), it translocates to the nucleus where it can activate the expression of AG target

genes. The induction of AG function in this line results in flowers with functional stamens and carpels, which resemble the flowers of 35S::AG plants (Ito *et al*., 2004).

After DEX treatment, *p35S::AG-GR*, *ag* plants carrying the *pREM22::GUS-GFP* construct display the development of wild-type stamen structures, as previously shown (Gomez-Mena *et al*., 2005, Ito *et al*., 2007, Ito *et al*., 2004). Confocal-based live imaging of these plants at stage 6 of flower development showed that GFP expression is very similar to that observed in *pREM22::GUS-GFP* plants (Fig. 4 A-C), though the GFP signal is weaker and more diffuse. The induction of these plants with DEX activates *NZZ/SPL* exclusively in lateral edges of the distal parts of organ primordial giving rise to a petal with one locule on each either side (Ito *et al*., 2004). In agreement with this, at later stages of development, GFP expression is observed in the middle cell layer and tapetal cells of all staminoid petals (Fig. 4 D-F). Therefore, these results indicate that stamen differentiation proceeds normally in *p35S::AG-GR, ag* plants and furthermore, that the line *pREM22::GUS-GFP* is a valuable marker for tracing stamen histodifferentiation. In *pREM22::GUS-GFP, ag* plants, we observed low levels of GFP expression in L1 and L2 cells (Fig. 4 J-L) at stage 5 of flower development. This data suggests that early expression of *REM22* is independent of AG function.

Discussion

The present work provides a detailed analysis of *REM22* mRNA expression. The *in situ* hybridization analysis and the confocal-based live imaging study of *pREM22::GUS-GFP* plants revealed that the fragment of 1022 bp contains the majority of the control elements necessary to stamen expression. The unique expression pattern of *REM22* during early stamen development makes it a potential candidate for a stamen marker. In order to demonstrate the usefulness of the *pREM22::GUS-GFP* plants we crossed it to the line *p35S::AG-GR, ag*. The results support the concept that this line can be useful to identify the anther primordial position. The promoter activity of *REM22* is also observed in L1 and L2 of carpel primordia only at stage 8, when the key events of stamen differentiation such as stamen identity, archeosporial initiation and parietal and microspore precursor formation have already taken place. After stage 10, *REM22* expression is observed during ovule development when its expression is off in stamens. Although, the *REM22* promoter activity in carpels may hinder its usefulness at later flower stages, its high specific expression at early stages of stamen development can be very useful for characterization of novel mutant backgrounds with stamen identity problems or with defects in stamen differentiation at early stages. Our data also suggests that early expression of *REM22* is independent of AG function. Based on electrophoretic mobility shift assays (EMSA), it has been proposed that the AG protein binds to a putative CArG box located at position -1570 bp of the *REM22* promoter (Gomez-Mena *et al*., 2005). However, since our *pREM22::GUS-GFP* only contains sequences from -1 to -1022 bp and does not contain the putative CArG box, it is likely that the work described here does not evaluate the influence of this putative *cis* element on the *REM22* expression pattern. Equally, it is possible that the EMSA results are artifactual, since it is not an *in vivo* assay.

The lack of a mutant phenotype in the *REM22* knock out line is likely a consequence of the high degree of functional redundancy found in *Arabidopsis*, especially among member of REM family (Romanel *et al*., 2009). In fact, there are two *REM22* close homologues in the *Arabidopsis* genome, *REM20* and *REM21* (Romanel *et al*., 2009). *REM20*, named *VERDANDI*, was recently identified and the characterization of female gametophytes in the T-DNA insertion line indicates that antipodal and synergid cell identity and/or differentiation are affected (Matias-Hernandez *et al*., 2010).

REM22 expression, when compared to other marker genes already characterized during reproductive development, is unique. It has an expression restricted to few cells, the precursors of stamen primordia, in the floral meristem when *APETALA1* (*AP1*)

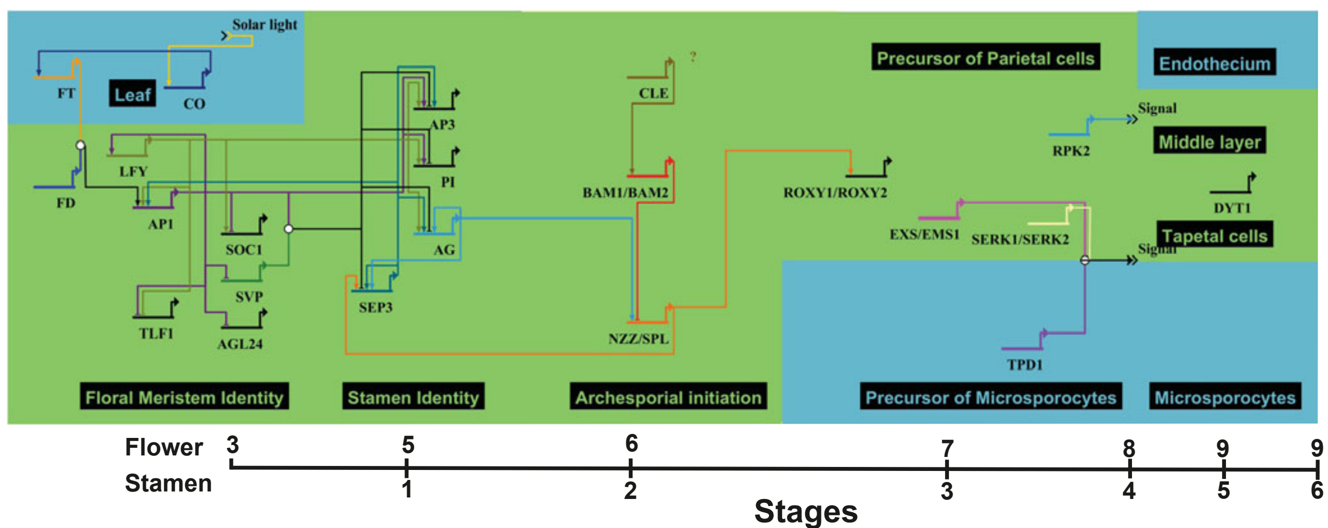

Fig. 5. Gene regulatory network controlling early stages of stamen development in *Arabidopsis thaliana*. *Genes that respond to circadian clock genes, floral-promotion genes, floral meristem identity genes, stamen identity genes and genes necessary for stamen tissue differentiation are shown. Individual genes are represented by horizontal lines with bent arrows and gene symbols. For each gene, upstream inputs and downstream targets are indicated. Activators are connected to their target by arrow, repressors by blunted lines. White circles represent protein interactions. Arrows with "signal" symbolize that gene or protein interactions are responsible for triggering the development of the next step. The green area represents the expression domain of the* REM22 *marker gene. Diagram was generated using BioTapestry (Longabaugh* et al.*, 2005) and is based on published data (see text).*

and *LEAFY* (*LFY*) RNAs accumulate uniformly throughout young floral primordia (Bowman *et al.*, 1993, Mandel *et al.*, 1992, Weigel *et al.*, 1992). Later, at stage 5, when the expression of ABCE genes is observed, *REM22* is expressed exclusively in the L1 and L2 of only in the third whord. *AP3* transcripts accumulate in the precursors of stamen, but also precursors of petals prior to the differentiation of these organs (Jack *et al.*, 1992). During stamen tissue differentiation, *REM22* expression is restricted to archesporial cells where is also expressed *BAM1*/*BAM2* and *NZZ* (Hord *et al.*, 2006, Schiefthaler *et al.*, 1999). At stage 7, *ROXY1*/*ROXY2* (Xing and Zachgo, 2008), *BAM1*/*BAM2* and *EXS*/*EMS1* RNA is limited to the primary parietal and primary sporogenous cells near the lateral edges of anthers whereas *NZZ* is broadly expressed in stamen tissues. From stages 3 to 8, *REM22* RNA expression is distinctive when compared with *ROXY1*/*ROXY2*, *EXS1*/*EMS1*, *SERK1*/*SERK2* and *RPK2*, been restricted to primary parietal cells and inner secondary parietal cells at stage 8 (Fig. 5) (Canales *et al.*, 2002, Colcombet *et al.*, 2005, Xing and Zachgo, 2008, Zhao *et al.*, 2002). Finally, with the differentiation of the endothecium, middle layer, tapetal cells and microspores, *REM22* expression is similar to *SERK1*/*SERK2* (Colcombet *et al.*, 2005), restricted to the middle layer and tapetum tissue.

The *REM22* promoter can also be a useful tool for genome wide tissue/cell specific expression studies, or translatome analysis, as well as chromatin immunoprecipitation assays for those interested in mechanisms of transcriptional regulation during early tissue differentiation in stamens. Moreover, its association with the floral induction system in *Arabidopsis* developed by Wellmer and collaborators that allows the isolation of a large number of synchronized floral buds in a specific floral stage, could make it more sensitive by enriching the biological sample with particular tissue types of a specific developmental stage (Wellmer *et al.*, 2006).

Materials and Methods

Plant material, growth conditions

The *Arabidopsis Thaliana* Landsberg Erecta (Ler) was used as wild-type control for plants transformed with constructed vector. The *A. thaliana* Columbia (Col) was used as wild-type control for plants carrying T-DNA insertion. The *rem22-2* (SALK_091149) insertion line was isolated from Salk collection (http://signal.salk.edu) (Alonso *et al.*, 2003). The T-DNA line used in this work is in Col background. Seeds were stored at 4ºC for all the time. To conduct the experiments, seeds from Salk collection were surface rinsed and sterilized with ethanol 100% for 5 minutes, and then germinated in soil under short (16Hs dark/8Hs light) and long-day conditions (16Hs light/8Hs dark) at 19 to 23ºC. Seeds containing constructed plasmid were surface sterilized with ethanol 70% for 5 minutes, rinsed with ethanol 100%, and then germinated and grown on Murashige and Skoog medium (Murashige and Skoog, 1962) under long-day conditions at 19 to 23ºC. After two weeks, seedlings were transferred to a mix of vermiculite and soil at the same conditions for growth.

Genotyping and gene expression analysis

Segregation analysis and genotyping were applied to isolate lines homozygous for a T-DNA insertion. Primers LP (5'-TTGAAAAGTTAGATCTTTCAGCTTTG-3') and RP (5'-TATGGTTTGAATCTTACCAGGAACT-3') were used to amplify a wild-type or an insertion allele of a gene in combination with the T-DNA-specific primer LBb1 (5#- CAAACCAGCGTGGACCGCTTGCTG -3´). The exact positions of T-DNA insertion in the line SALK_091149 (target gene At3g17010) was determined by sequencing of the resulting PCR products. Total RNA was isolated with the RNeasy RNA isolation kit followed by DNase I treatment (Qiagen) from 100 mg of whole inflorescences of REM22 knock out line and wild-type plants, respectively. cDNA was generated from two independent RNA preparations for each genotype using MLV-RT First-Strand Synthesis System (Promega) according to the manufacturer's instructions. Primers used for RT-PCR (Supplementary Table 1) were designed to amplify 400- to 450-bp (RT-PCR) long fragments of cDNA. *UBQ10* (AT4G05320) was used to normalize the mRNA sources (Michaels *et al.*, 2004). For PCR, reactions were carried out in a total volume of 25 μL with 0.4 mM primers and 200 mM dNTPs using Ex-Taq polymerase (Takara) according to the manufacturer's instructions. PCR conditions were as follows: 94°C for 4 min; 20 to 35 cycles of 94°C for 30 s, 55°C for 1 min, 72°C for 1 min; followed by an elongation step at 72°C for 10 min. The amplification products were visualized on a 1% (w/v) agarose gel via ethidium bromide staining.

Plasmid construct and generation of transgenic plants

To obtain the complete promoter region from *At3g17010* (*REM22*), the 1022 bp upstream ATG was cloned into a binary vector pKGWFS7 (GUS/GFP) using the Gateway cloning technology and sequenced to confirm the fidelity of the promoter sequences. The *pREM22::GUS-GFP* construct was introduced into the *Agrobacterium tumefaciens* strain GV3101 and transformed into *Arabidopsis Thaliana* wild-type Ler by the floral dip method (Clough and Bent, 1998). T2 lines segregating for kanamycin resistance (25 μg/mL) with an approximately 3:1 ratio were selected for further analysis.

DEX treatment and **pREM22::GUS-GFP** *in* **ag 35S:AG-GR** *background*

Plants carrying *p35S::AG-GR*, *ag* background (Ito *et al.*, 2004) were treated every day for one week by submerging the inflorescences in a solution containing 10uM DEX together with 0.015% Silwet L-77 for ~ 1 min. One recovered transgenic *p35S::AG-GR*, *ag* line was crossed with *pREM22::GUS-GFP* plants to obtain lines in subsequent generations. F2 generation inflorescences of *ag-1 35S::AG-GR*/*pREM22::GUS-GFP* plants with *agamous* phenotypes were chosen to genotype with GUS primer 5' – GCGACGCTCACACCGATAC – 3' and 5' – ACTGCTTTTTCTTGCCGTTTTC – 3' and GR primer 5' – ATGGCTAGTGAAGCTCGAAAAACA - 3' and 5' – TTTTTGATGAAACAGAAGCTT– 3'. Plants positive for both primers were used in the experiment. Some control plants were mock-treated and some plants were continuously treated with DEX for 5 days once a day. These plants were observed by live imaging.

Tissue preparation for live imaging

The presence of the GFP protein was determined in primary inflorescence containing floral buds of stage 1-12 living tissue. They were prepared by cutting off flowers older than stage 13. These inflorescences were transferred into clear plastic boxes containing MS-agar. We applied 10 μg ml–1 FM4-64 (Molecular Probes), a water-soluble lipophilic dye, directly onto the inflorescence meristem 30 min prior to imaging. Confocal laser scanning microscopy of the living plant tissue was performed with Zeiss 510L SM Meta with water dipping 63x objective achroplan lens. Stacks of 30 sections spaced approximately 1.5 μm apart were collected every 2 hr. Visualization of sections and volume was performed using the Zeiss LSM software.

Histochemical GUS Staining and histological analysis

Inflorescence plants were collected and placed in 90% ice-cold acetone for 30 min, washed in staining 0.1 M phosphate buffer, pH7.0, and incubated overnight at 37ºC in buffer with 2 mM X-Gluc substrate, 1mM potassium ferricyanide, and 1 mM potassium ferrocyanide. The plant material was stored in 70% ethanol until analyzed.

Probe making and **in situ** *hybridization*

Constructs used for probes generation and oligonucleotide sequences are described earlier (Alves-Ferreira *et al.*, 2007). All the steps of *in situ* hybridization were carried out according to the protocol described by Alves-Ferreira and collaborators (2007).

Accession numbers

Arabidopsis Genome Initiative locus identifiers of *Arabidopsis* genes used in this article are as follow: AG (At4g17010), UBQ (At4g05320) and REM22 (At3g17010).

Acknowledgements

E.R. thanks Annick Dubois and Pradeep Das for friendship and hosting assistance to develop this work; to Robert Franks for comments on the manuscript; and to Marcus Heisler for initial analysis of REM22 marker line by confocal microscopy. M.A.-F. was supported by grants from CNPq and Fundação de Amparo à Pesquisa do Rio de Janeiro (FAPERJ; M. Alves--Ferreira: #E-26/102.861/2008). This work is part of E.R.`PhD thesis from Department of Genetics of the Universidade Federal do Rio de Janeiro, Brazil. E.R. was supported by a PhD studentship (Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and UNESCO.

References

ALBRECHT, C., RUSSINOVA, E., HECHT, V., BAAIJENS, E. and DE VRIES, S. (2005). The *Arabidopsis Thaliana* SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES1 and 2 control male sporogenesis. *Plant Cell* 17: 3337-3349.

ALVES-FERREIRA, M., WELLMER, F., BANHARA, A., KUMAR, V., RIECHMANN, J.L. and MEYEROWITZ, E.M. (2007). Global expression profiling applied to the analysis of *Arabidopsis* stamen development. *Plant Physiol.* 145: 747-762.

BOWMAN, J.L., ALVAREZ, J., WEIGEL, D., MEYEROWITZ, E.M. and SMYTH, D.R. (1993). CONTROL OF FLOWER DEVELOPMENT IN *ARABIDOPSIS*-THALIANA BY APETALA1 AND INTERACTING GENES. *Development* 119: 721-743.

CANALES, C., BHATT, A.M., SCOTT, R. and DICKINSON, H. (2002). EXS, a putative LRR receptor kinase, regulates male germline cell number and tapetal identity and promotes seed development in *Arabidopsis. Curr. Biol.* 12: 1718-1727.

CLOUGH, S.J. and BENT, A.F. (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis Thaliana. Plant J.* 16: 735-743.

COEN, E.S. and MEYEROWITZ, E.M. (1991). The War of the Whorls - Genetic Interactions Controlling Flower Development. *Nature* 353: 31-37.

COLCOMBET, J., BOISSON-DERNIER, A., ROS-PALAU, R., VERA, C.E. and SCHROEDER, J.I. (2005). *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR KINASES1 and 2 are essential for tapetum development and microspore maturation. *Plant Cell* 17: 3350-3361.

DITTA, G., PINYOPICH, A., ROBLES, P., PELAZ, S. and YANOFSKY, M.F. (2004). The SEP4 gene of *Arabidopsis Thaliana* functions in floral organ and meristem identity. *Curr. Biol.* 14: 1935-1940.

FRANCO-ZORRILLA, J.M., CUBAS, P., JARILLO, J.A., FERNANDEZ-CALVIN, B., SALINAS, J. and MARTINEZ-ZAPATER, J.M. (2002). AtREM1, a member of a new family of B3 domain-containing genes, is preferentially expressed in reproductive meristems. *Plant Physiol.* 128: 418-427.

GOMEZ-MENA, C., DE FOLTER, S., COSTA, M.M.R., ANGENENT, G.C. and SABLOWSKI, R. (2005). Transcriptional program controlled by the floral homeotic gene AGAMOUS during early organogenesis. *Development* 132: 429-438.

GOTO, K., KYOZUKA, J. and BOWMAN, J.L. (2001). Turning floral organs into leaves, leaves into floral organs. *Curr. Opin. Genet. Dev.* 11: 449-456.

HORD, C.L.H., CHEN, C.B., DEYOUNG, B.J., CLARK, S.E. and MA, H. (2006). The BAM1/BAM2 receptor-like kinases are important regulators of *Arabidopsis* early anther development. *Plant Cell* 18: 1667-1680.

ITO, T., NG, K.H., LIM, T.S., YU, H. and MEYEROWITZ, E.M. (2007). The homeotic protein AGAMOUS controls late stamen development by regulating a jasmonate biosynthetic gene in *Arabidopsis. Plant Cell* 19: 3516-3529.

ITO, T., WELLMER, F., YU, H., DAS, P., ITO, N., ALVES-FERREIRA, M., RIECHMANN, J.L. and MEYEROWITZ, E.M. (2004). The homeotic protein AGAMOUS controls microsporogenesis by regulation of SPOROCYTELESS. *Nature* 430: 356-360.

JACK, T., BROCKMAN, L.L. and MEYEROWITZ, E.M. (1992). THE HOMEOTIC GENE APETALA3 OF *ARABIDOPSIS*-THALIANA ENCODES A MADS BOX AND IS EXPRESSED IN PETALS AND STAMENS. *Cell* 68: 683-697.

KAGAYA, Y., OHMIYA, K. and HATTORI, T. (1999). RAV1, a novel DNA-binding protein, binds to bipartite recognition sequence through two distinct DNA-binding domains uniquely found in higher plants. *Nucl. Acids Res.* 27: 470-478.

KAUFMANN, K., MUINO, J.M., JAUREGUI, R., AIROLDI, C.A., SMACZNIAK, C., KRAJEWSKI, P. and ANGENENT, G.C. (2009). Target Genes of the MADS Transcription Factor SEPALLATA3: Integration of Developmental and Hormonal Pathways in the *Arabidopsis* Flower. *PLoS Biology* 7: 854-875.

LEVY, Y.Y., MESNAGE, S., MYLNE, J.S., GENDALL, A.R. and DEAN, C. (2002). Multiple roles of *Arabidopsis* VRN1 in vernalization and flowering time control. *Science* 297: 243-246.

LONGABAUGH, W.J.R., DAVIDSON, E.H. and BOLOURI, H. (2005). Computational representation of developmental genetic regulatory networks. *Dev. Biol.* 283: 1-16.

MANDEL, M.A., GUSTAFSONBROWN, C., SAVIDGE, B. and YANOFSKY, M.F. (1992). MOLECULAR CHARACTERIZATION OF THE *ARABIDOPSIS* FLORAL HOMEOTIC GENE APETALA1. *Nature* 360: 273-277.

MATIAS-HERNANDEZ, L., BATTAGLIA, R., GALBIATI, F., RUBES, M., EICHENBERGER, C., GROSSNIKLAUS, U., KATER, M.M. and COLOMBO, L. (2010). VERDANDI Is a Direct Target of the MADS Domain Ovule Identity Complex and Affects Embryo Sac Differentiation in *Arabidopsis. Plant Cell* 22: 1702-1715.

MELZER, R. and THEISSEN, G. (2009). Reconstitution of floral quartets *in vitro* involving class B and class E floral homeotic proteins. *Nucleic Acids Res.* 37: 2723-2736.

MELZER, R., VERELST, W. and THEISSEN, G. (2009). The class E floral homeotic protein SEPALLATA3 is sufficient to loop DNA in floral quartet-like complexes in vitro. *Nucleic Acids Res.* 37: 144-157.

MIZUNO, S., OSAKABE, Y., MARUYAMA, K., ITO, T., SATO, T., SHINOZAKI, K. and YAMAGUCHI-SHINOZAKI, K. (2007). RPK2 is a novel factor controlling anther development in *Arabidopsis Thaliana. Plant Cell Physiol.* 48: S229-S229.

MURASHIGE, T. and SKOOG, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* 15: 473-497.

PELAZ, S., DITTA, G.S., BAUMANN, E., WISMAN, E. and YANOFSKY, M.F. (2000). B and C floral organ identity functions require SEPALLATA MADS-box genes. *Nature* 405: 200-203.

ROMANEL, E.A.C., SCHRAGO, C.G., COUÃ±AGO, R.M., RUSSO, C.A.M. and ALVES-FERREIRA, M.R. (2009). Evolution of the B3 DNA Binding Superfamily: New Insights into REM Family Gene Diversification. *PLoS ONE* 4: e5791.

SANDERS, P.M., BUI, A.Q., WETERINGS, K., MCINTIRE, K.N., HSU, Y.C., LEE, P.Y., TRUONG, M.T., BEALS, T.P. and GOLDBERG, R.B. (1999). Anther developmental defects in *Arabidopsis Thaliana* male-sterile mutants. *Sexual Plant Reprod.* 11: 297-322.

SCHIEFTHALER, U., BALASUBRAMANIAN, S., SIEBER, P., CHEVALIER, D., WISMAN, E. and SCHNEITZ, K. (1999). Molecular analysis of *NOZZLE*, a gene involved in pattern formation and early sporogenesis during sex organ development in *Arabidopsis Thaliana. Proc. Natl. Acad. Sci. USA* 96: 11664-11669.

SMYTH, D.R., BOWMAN, J.L. and MEYEROWITZ, E.M. (1990). Early Flower Development in *Arabidopsis. Plant Cell* 2: 755-767.

SUZUKI, M., KAO, C.Y. and MCCARTY, D.R. (1997). The Conserved B3 Domain of VIVIPAROUS1 Has a Cooperative DNA Binding Activity. *Plant Cell* 9: 799-807.

SUZUKI, M., WANG, H.H.Y. and MCCARTY, D.R. (2007). Repression of the LEAFY COTYLEDON 1/B3 Regulatory Network in Plant Embryo Development by VP1/ABSCISIC ACID INSENSITIVE 3-LIKE B3 Genes. *Plant Physiol.* 143: 902-911.

TSUKAGOSHI, H., SAIJO, T., SHIBATA, D., MORIKAMI, A. and NAKAMURA, K. (2005). Analysis of a sugar response mutant of *Arabidopsis* identified a novel B3 domain protein that functions as an active transcriptional repressor. *Plant Physiol.* 138: 675-685.

ULMASOV, T., HAGEN, G. and GUILFOYLE, T.J. (1997). ARF1, a transcription factor that binds to auxin response elements. *Science* 276: 1865-1868.

WEIGEL, D., ALVAREZ, J., SMYTH, D.R., YANOFSKY, M.F. and MEYEROWITZ, E.M. (1992). LEAFY CONTROLS FLORAL MERISTEM IDENTITY IN *ARABIDOPSIS. Cell* 69: 843-859.

WELLMER, F., ALVES-FERREIRA, M., DUBOIS, A., RIECHMANN, J.L. and MEYEROWITZ, E.M. (2006). Genome-wide analysis of gene expression during early *Arabidopsis* flower development. *Plos Genetics* 2: 1012-1024.

WIJERATNE, A.J., ZHANG, W., SUN, Y.J., LIU, W.L., ALBERT, R., ZHENG, Z.Q., OPPENHEIMER, D.G., ZHAO, D.Z. and MA, H. (2007). Differential gene expression in *Arabidopsis* wild-type and mutant anthers: insights into anther cell differentiation and regulatory networks. *Plant J.* 52: 14-29.

XING, S. and ZACHGO, S. (2008). ROXY1 and ROXY2, two *Arabidopsis* glutaredoxin genes, are required for anther development. *Plant J.* 53: 790-801.

YANG, S.L., XIEA, L.F., MAO, H.Z., PUAH, C.S., YANG, W.C., JIANG, L.X., SUNDARESAN, V. and YE, D. (2003). TAPETUM DETERMINANT1 is required for cell specialization in the *Arabidopsis* anther. *Plant Cell* 15: 2792-2804.

YANG, W.-C., YE, D., XU, J. and SUNDARESAN, V. (1999). The *SPOROCYTELESS* gene of *Arabidopsis* is required for initiation of sporogenesis and encodes a novel nuclear protein. *Genes Dev.* 13: 2108-2117.

ZHANG, W., SUN, Y., TIMOFEJEVA, L., CHEN, C., GROSSNIKLAUS, U. and MA, H. (2006). Regulation of *Arabidopsis* tapetum development and function by DYSFUNCTIONAL TAPETUM1 (DYT1) encoding a putative bHLH transcription factor. *Development* 133: 3085-3095.

ZHAO, D.-Z., WANG, G.-F., SPEAL, B. and MA, H. (2002). The EXCESS MICROSPOROCYTES1 gene encodes a putative leucine-rich repeat receptor protein kinase that controls somatic and reproductive cell fates in the *Arabidopsis* anther. *Genes Dev.* 16: 2021-2031.

Further Related Reading, published previously in the *Int. J. Dev. Biol.*

Multi-probe in situ hybridization to whole mount Arabidopsis seedlings
Leonardo Bruno, Antonella Muto, Natasha D. Spadafora, Domenico Iaria, Adriana Chiappetta, Mieke Van Lijsebettens and Maria B. Bitonti
Int. J. Dev. Biol. (2011) 55: 197-203

Genetic analysis of reproductive development in tomato
Rafael Lozano, Estela Giménez, Beatriz Cara, Juan Capel and Trinidad Angosto
Int. J. Dev. Biol. (2009) 53: 1635-1648

Chromatin remodeling in plant development
José A. Jarillo, Manuel Piñeiro, Pilar Cubas and José M. Martínez-Zapater
Int. J. Dev. Biol. (2009) 53: 1581-1596

Plant Developmental Biology in Spain:
from the origins to our days and prospects for the future
José-Pío Beltrán
Int. J. Dev. Biol. (2009) 53: 1219-1234

Long-range signalling in plant reproductive development
Paula Suárez-López
Int. J. Dev. Biol. (2005) 49: 761-771

The making of gametes in higher plants
Leonor C. Boavida, Jörg D. Becker and José A. Feijó
Int. J. Dev. Biol. (2005) 49: 595-614

5 yr ISI Impact Factor (2010) = 2.961