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Research article

Identification of two highly specific pollen promoters using transcriptomic data

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ABSTRACT

The mature pollen grain displays a highly specialized function in angiosperms. Accordingly, the male gametophyte development involves many specific biological activities, making it a complex and unique process in plants. In order to accomplish this, during pollen development, a massive transcriptomic remodeling takes place, indicating the switch from a sporophytic to a gametophytic program and involving the expression of many pollen specific genes. Using microarray databases we selected genes showing pollen-specific accumulation of their mRNAs and confirmed this through RT-PCR. We selected five genes (*POLLEN SPECIFIC GENE1-5*) to investigate the pollen specificity of their expression. Transcriptional fusions between the putative promoters of these genes and the uidA reporter gene in Arabidopsis confirmed the pollen specific expression for at least two of these genes. The expression of the cytotoxin *Barnase* controlled by these promoters generated pollen specific ablation and male sterility. Through the selection of pollen specific genes from public datasets, we were able to identify promoter regions that confer pollen expression. The use of the cytotoxin Barnase allowed us to demonstrate its expression is exclusively limited to the pollen. These new promoters provide a powerful tool for the expression of genes exclusively in pollen.

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1. Introduction

Pollen grains of angiosperms have the important and specialized function that allows plant sexual reproduction. To accomplish its functions, the male gametophyte has to undergo a complex developmental program, that involves many important biological processes, such as changes in cell polarity, cell wall biogenesis, mitosis and cell fate determination (McCormick, 2004), all these supported on a unique gene expression program. Pollen development starts after meiosis, when the uninucleated microspores (UNM) are produced within the anther. Then, a highly asymmetric mitosis takes place and two very different cells are formed: a large vegetative cell and a small generative cell, producing bicellular pollen grains (BCP). In Arabidopsis and other species, the generative cell undergoes a second mitosis, producing to sperm cells inside the vegetative cell and giving rise to tricellular pollen grains (TCP). During this process, pollen grains accumulate starch and lipids to sustain pollen tube growth during fertilization and finally, pollen grains dehydrate, enter in a quiescent state and are denominated mature pollen grains (MPG) (McCormick, 2004).

Transcriptomic studies performed in pollen of *Arabidopsis thaliana*, revealed that nearly 14,000 genes are expressed throughout pollen development, and 5% of these genes are specific to this process (Honys and Twell, 2004; Pina et al., 2005; Zimmermann et al., 2004). Additionally, studies performed in pollen at different developmental stages showed that most of these genes are expressed during the initial stages. In contrast, the amount of pollen-specific genes expressed increases during male gametophyte development, with a maximum in mature pollen grains, suggesting a specialization of the transcriptome during this process (Borg et al., 2009).

Many promoters have been described as pollen or anther specific in several species (Khurana et al., 2012). The best characterized is the late pollen specific family (*LAT*). *LAT* promoters from *Lycopersicum esculentum* have been studied and regulatory sequences that confer the pollen specific expression have been identified (Twell et al., 1989; Eyal et al., 1995). The maize *Zm13* gene was the first pollen specific gene described (Hamilton et al., 1989; Hanson et al., 1989) and the regulatory region activates expression in the microspore mitosis during microgametophyte development

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(Guerrero et al., 1990). In both cases (and others) the pollen specific expression has been studied using *GFP* and/or *GUS* as reporter genes.

The available transcriptomic data obtained by microarrays analysis provides a powerful tool to identify pollen-specific genes, and hence facilitates the identification of regulatory sequences directing pollen-specific expression. Here we report the identification of five genes with highly specific pollen expression. Promoters of these five genes were used to drive *GUS* expression in transgenic Arabidopsis plants. Two of these promoters were selected to drive the expression of the RNase *Barnase*. Pollen ablation and male sterility was observed in these transgenic lines, confirming the highly specificity of these promoters.

2. Materials and methods

2.1. Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia (Col-0) plants were grown hydroponically, under a 16-h light/8-h dark cycle, at 23 °C (Gibeaut et al., 1997). Seeds were surface sterilized for 6 min with a solution of 50% (v/v) commercial bleach, and then washed with sterile distilled water. Seeds were germinated and grown on half-strength Murashige and Skoog (MS) medium (Phytotechnology Laboratories) with 0.8% (w/v) agar and supplemented with kanamycin (50 mg/L) if necessary. *Arabidopsis thaliana* ecotype Columbia plants were transformed with Agrobacterium by floral dip (Clough and Bent, 1998). T_1 seeds were sown on half-strength MS medium with 0.8% (w/v) agar supplemented with kanamycin (50 mg/L). T_2 lines were tested for a 1:3 sensitive:insensitive segregation on MS plates supplemented with kanamycin.

2.2. Selection of candidate genes

Selection of candidate genes was performed as described previously (Lucca and León, 2012). Briefly, using the microarray data from pollen development (Honys and Twell, 2004), we selected genes that showed progressive expression, starting at BCP and peaking at TCP or MPG. Then, microarray data from vegetative tissues (Schmid et al., 2005) were used to select candidate genes for pollen-specific expression by choosing genes that only have expression in flower stages 12 and 15, stamen, anther and mature pollen grains. Finally, the five genes showing the highest pollen to sporophytic mRNA accumulation ratio were selected.

2.3. RT-PCR analyses

Total RNA was isolated from different tissues using TRIzol reagent (Invitrogen) For RNA extraction from mature pollen grains we used the RNeasy Plant Mini Kit (Qiagen) and cDNA was obtained using the SuperScript III First-Strand Synthesis System (Invitrogen). cDNA was amplified using primers that flanked an intron for each *PSG* (Supplemental Table 1). Clathrin adaptor complex subunit (At5g46630) was used as control (Czechowski et al., 2005).

2.4. Generation of PSG_{pro}:GUS and PSG_{pro}:Barnase constructs

For the generation of the *PSG_{pro}:GUS* constructs, the upstream intergenic region of each *PSG* was amplified using specific primers (Supplemental Table 1), cloned into pENTR/D/TOPO (Invitrogen) and sequenced. The cloned upstream intergenic regions were then recombined into pKGWFS7 (VIB, Ghent, Belgium) destination vector using the LR Clonase enzyme mix (Invitrogen). The *PSG_{pro}:Barnase* constructs were generated using GATEWAY multisite cloning system (Invitrogen). We performed an amplification of each upstream intergenic region of the *PSG* using specific primers (Supplemental Table 1) and cloned into the pENTR5'/TOPO entry vector (Invitrogen). The *Barnase* coding sequence was amplified from the pMT416 plasmid (Hartley, 1988) with primers (Supplemental Table 1) producing a 362 bp size sequence that was cloned into the pCR8 entry vector (Invitrogen) and sequenced. Then, a multisite recombination was performed using the LR Clonase Plus II enzyme mix (Invitrogen), between both entry vectors and pK7m2,4 GW destination vector. The generated constructs were transformed into *Agrobacterium tumefaciens* GV3101.

2.5. Histochemical GUS activity

GUS activity was adapted from (Jefferson et al., 1987). In brief, tissues of *PSG_{pro}:GUS* transgenic plants were incubated for 16 h at 37 °C in GUS buffer (100 mM NaH_2PO_4, pH 7.2; 10 mM EDTA, pH 8.0; 10% [v/v] methanol; 0.3% Triton X-100; 0.5 mM $K_3Fe[CN]_6$; 0.5 mM $K_4Fe[CN]_6$) supplemented with X-Gluc 1 mM. Samples were washed with 70% ethanol until cleared and viewed using an Olympus SZX7 stereoscopic microscope (Olympus). Images were captured using a Cannon PowerShot A640 camera (Cannon).

2.6. Microscopic analyses

Alexander staining of pollen grains was performed as described before (León et al., 2007). For histological analyses of pollen and anthers, flowers at different developmental stages were fixed overnight (3% [v/v] glutaraldehyde, 0.1 M sodium cacodylate [pH 7.2]), dehydrated in acetone series to 100% and embedded in Embed 812 resin. Anther transverse sections (2 μm) were stained with 1% safranin for GUS analyses in pollen or 1% toluidine blue for pollen analyses in transgenic plants expressing *Barnase*. Anthers cross-sections were viewed using an Olympus IX81 microscope (Olympus), bright-field photographs were taken using a Micro-Publisher 3.3 RTV digital and images were processed using Adobe Photoshop CS5.

3. Results

3.1. Identification of pollen specific candidate genes

Using the normalized *Arabidopsis thaliana* microarrays database available at AVT (AtGenExpress Visualization Tool, Schmid et al., 2005) and the pollen transcriptome database (Honys and Twell, 2004) we searched for pollen-specific genes using the following criteria: transcripts should only be present post-meiotically, starting from the bicellular pollen grain stage (Fig. 1A), and should be specific for pollen or tissues that contain pollen (flower and stamen, Fig. 1B). We made a selection of 50 genes, and from these we selected the top five genes that show the highest ratio of transcripts in pollen compared to sporophytic tissues and the selected genes were named *PSG* for *POLLEN SPECIFIC GENES*. *PSG1* (At3g01620, annotated as a β-1,4-N-acetylglucosaminyltransferase family protein), *PSG2* (At1g28550, annotated as a RAB GTPase homolog A1I), *PSG3* (At1g03840, annotated as MGP, a nuclear-localized putative transcription factor with three zinc finger domains), *PSG4* (At4g27110, annotated as a COBRA-like protein 11 precursor) and *PSG5* (At2g40990, annotated as a DHHC-type zinc finger family protein). The physiological relevance of these genes in the context of pollen development has been studied through the analysis of insertional mutant lines. No defects in pollen development have been observed (unpublished data from our laboratory). Pollen-

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