

# Functional genomics of pollen tube–pistil interactions in *Arabidopsis*

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## Abstract

The pollen tube represents an attractive model system for functional genomic analysis of the cell–cell interactions that mediate guided cellular growth. The pollen tube extends through pistil tissues and responds to guidance cues that direct the tube towards an ovule, where it releases sperm for fertilization. Pollen is readily isolated from anthers, where it is produced, and can be induced to produce a tube *in vitro*. Interestingly, pollen tube growth is significantly enhanced in pistils, and pollen tubes are rendered competent to respond to guidance cues after growth in a pistil. This potentiation of the pollen tube by the pistil suggested that pollen tubes alter their gene-expression programme in response to their environment. Recently, the transcriptomes of pollen tubes grown *in vitro* or through pistil tissues were determined. Significant changes in the transcriptome were found to accompany growth *in vitro* and through the pistil tissues. Reverse genetic analysis of pollen-tube-induced genes identified a new set of factors critical for pollen tube extension and navigation of the pistil environment. Recent advances reviewed in the present paper suggest that functional genomic analysis of pollen tubes has the potential to uncover the regulatory networks that shape the genetic architecture of the pollen tube as it responds to migratory cues produced by the pistil.

## Introduction

Co-ordinated development in multicellular organisms involves cells responding to signals from neighbouring cells and their micro-environment. This is particularly important for migrating cells, which sense and respond to multiple cues that direct them to their destination. Cell–cell interactions are mediated by membrane receptors that bind ligands expressed by neighbouring cells. These cell–surface interactions are transduced to the nucleus via signal transduction pathways, resulting in gene-expression changes in responding cells. Isolating sufficient quantities of a single cell type to determine responses to developmental cues is very difficult and often requires invasive techniques; these challenges are compounded for migratory cells. The structural, developmental and functional attributes of the PTs (pollen tubes) of flowering plants coupled with recently developed protocols to collect sufficient quantities of pure PTs, make it an attractive model system for understanding the molecular basis of cell-type-specific developmental responses in a migrating cell.

Pollen development follows meiosis in specialized floral structures called anthers [1]. When pollen grains reach the pistil, cell–cell interactions between the pollen grain and stigmatic papillae lead to the formation of a PT. The tip-growing PT extends between the walls of the stigmatic cells, travels through an extracellular matrix in the transmitting tissue of the style and finally arrives at the ovary. In the ovary,

the PT enters the ovule, arrests growth in the embryo sac and releases two sperm cells for double fertilization, a process in which one sperm fertilizes the egg cell to generate the embryo and the other sperm cell fuses with the central cell to form the endosperm [2].

A dynamic gene-expression programme probably facilitates the PT response to multiple guidance cues and the ever-changing environment encountered during its migration through the pistil. Yet, the molecular, genetic and cellular bases of signal perception and the signalling networks that modulate PT growth and guidance are largely unknown. Recent advances in PT functional analysis promise to facilitate elucidation of the molecular basis of PT guidance. For instance, the number of pollen and PT-expressed genes has increased exponentially as a result of global RNA profiling studies [3–6]. A number of novel assays to characterize PT function have been developed [7,8]. Time-lapse imaging of pistil-dependent PT growth and double fertilization have been developed in *Arabidopsis thaliana* [8,9]. These developments, combined with the availability of a novel mutant population that facilitates quantitative phenotypic characterizations [7], promise to accelerate functional analysis of many PT-expressed genes. In the present paper, we review recent advances in PT gene discovery and discuss a comprehensive reverse genetic analysis scheme to explore the function of these genes.

## Discovery of genes critical for PT function by mutant screens

Mutations in pollen-expressed genes will not be transmitted efficiently to the next generation and therefore exhibit

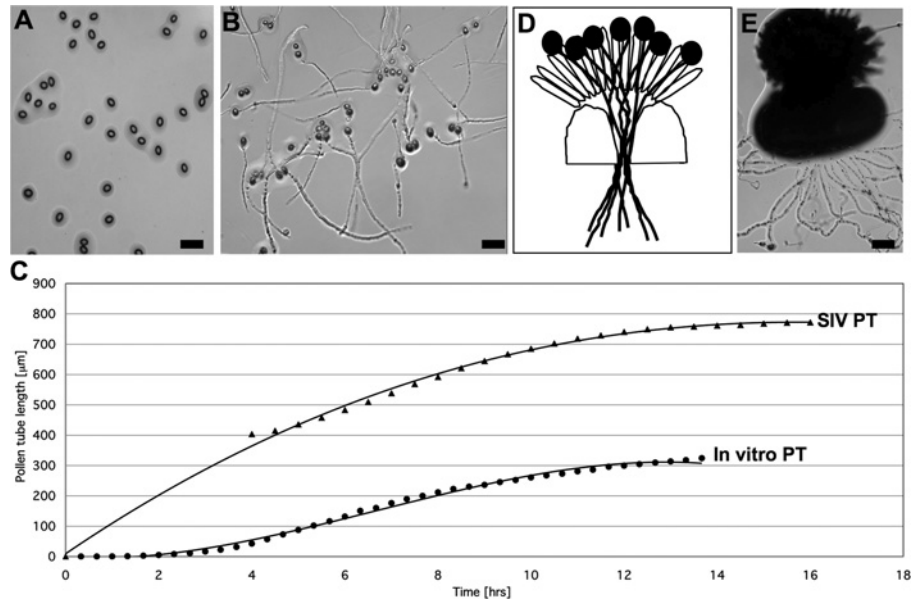
**Key words:** *Arabidopsis thaliana*, functional genomics, pistil, pollen tube.

**Abbreviations used:** GUS,  $\beta$ -glucuronidase; PT, pollen tube; SAIL, Syngenta *Arabidopsis* Insertion Library; SIV semi-*in vitro*; T-DNA, transferred DNA.

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**Figure 1 | *In vitro* and SIV PT growth in *Arabidopsis***

(A) Bright-field micrograph of ungerminated pollen deposited on pollen growth medium. (B) Bright-field micrograph of PT growth after incubation in pollen growth medium for 4 h. Many grains exhibit elongated tubes. (C) PT growth on a cut pistil (SIV PT) or *in vitro* (In vitro PT). The average growth rate of 12 PTs for each condition were plotted. Rapid growth was seen in the pistil during the first few hours, and after PTs exit the pistil and begin to grow on the pollen growth medium, their growth decreases as they move away from the pistil; nonetheless, the SIV PT growth rate remains higher than *in vitro* PT. The terminal length of SIV PT is also much longer than that of *in vitro* PT. (D) Diagram of SIV PT growth. Bundles of PTs that emerge from the style are excised for RNA isolation. (E) Light micrograph of SIV grown PT from cut pistil explant. Scale bars, 25  $\mu\text{m}$ .



non-Mendelian inheritance. Distorted segregation has been used as the basis of several screens to identify genes that are important for pollen development and function (for example, see [7,10–12]). To date, similar mutant screens have only identified very few PT-expressed genes [7,11–16]. Mutant screens alone may be insufficient to identify many of the genes that are important for pollen function. For example, mutations in many of the pollen-expressed genes may not result in a phenotype. In addition, mutant screens will not recover mutations in genes that are essential for development and/or function of both gametophytes. There is clearly a discrepancy between the number of pollen-expressed genes (~14 000 genes are expressed in at least one stage of pollen development [6]) and those identified by mutant screens. Therefore gene-expression profiling is a critical complementary approach that can reveal the basic genetic architecture of the PT.

### Characterization of *in vitro*-grown PT transcriptome

Mature pollen grains were thought to store the mRNA required for PT growth [17,18], so these mRNAs could be translated immediately following hydration [19]. However, it was recently established that new mRNAs, not present in the pollen grain, accumulate during PT growth [3,4]. Two microarray studies of total RNA isolated from

*Arabidopsis* PT grown *in vitro* for 0.5 h (Figure 1A) or 4 h (Figure 1B and Supplementary Movie S1 at <http://www.biochemsoctrans.org/bst/038/0380593add.htm>) showed that the number of transcripts and their abundance increased during pollen germination and growth [3,4]. Induced genes belonged to the gene ontology categories of cell rescue, transcription, signal transduction and cellular transport [3,4]. By comparing genes expressed during PT growth and sporophytic tissues, it was shown that a distinct set of genes are expressed exclusively during pollen hydration (0.5 h PT) and PT growth (4 h PT) [3]. These observations show that new and potentially significant transcription accompanies PT growth and raise an important question: does the PT transcriptome change as it grows through the pistil?

### Pistil-dependent PT capacitation

Many aspects of PT growth are similar regardless of whether PTs are grown *in vitro* or through pistil tissues. For example, polar growth and migration of the cytoplasm and sperm cells to the tip are observed under both conditions. Considerable differences have also been documented. PT length and the rate of PT elongation in a pistil is much higher than in an artificial growth medium (Figure 1C), perhaps due to the nutritional support provided by pistil tissues [18]. In addition, successful targeting of ovules by PTs is greatly improved if pollen germinates and grows through a pistil [8,20]. These

results indicate that pistil tissues transform PTs and capacitate them to perceive downstream signals from ovules. This potentiation of PTs by the pistil is functionally analogous to the transformation that mammalian spermatozoa undergo after they enter the female reproductive tract [21,22], and hence we refer to the PT transformation in the pistil as 'pollen tube capacitation'. It is unclear how pistil tissues modify the PT; nonetheless, it is expected that the transcriptomes of *in vitro*- and *in vivo*-grown PT would differ considerably and that a distinct set of PT transcripts would be expressed upon interaction with pistil tissues.

### PT grown by an SIV (semi-*in vivo*) procedure: a useful model system to study PT-pistil interactions

Isolation of RNA from pistil-grown PTs would allow determination of changes in the PT transcriptome in response to its native environment. Such isolation would be possible in species (for example, lily) with a pistil anatomy (hollow style) that facilitates isolation of intact PTs. The down side, at least for now, is that such species lack genomic resources. In species with solid styles, such as genomics resource-rich *Arabidopsis*, the drawback is that PTs adhere to the transmitting tract extracellular matrix, making it difficult to obtain *in vivo*-grown PTs of high purity without contamination from the surrounding pistil tissues. To overcome these challenges and to analyse the transcriptome of PTs that had interacted with pistil tissues, protocols to collect a large amount of *Arabidopsis* PTs grown by the SIV method (Figure 1D) were developed recently [3,8]. In this procedure, the upper portion of a pollinated pistil (stigma and style) is excised and placed horizontally on pollen growth medium. At 3 h after pollination, PTs emerge from the cut pistil and fan out on the solid pollen growth medium (Figure 1D and Supplementary Movie S2 at <http://www.biochemsoctrans.org/bst/038/0380593add.htm>).

The SIV PT procedure yields pure PTs without contamination from pistil tissue that can be used for RNA isolation without the need for additional manipulations (cell sorting or protoplast isolation). The procedure can be completed with wild-type PT; mutant or marker lines that facilitate isolation of a single cell type, but that might alter gene expression, are not required. The portion of tubes excised after they grow out of the pistil comprises the actively growing PT tip and the two sperm cells, and thus the SIV PT method enriches for the key components of a PT. Using the SIV PT system, the *Arabidopsis* pistil-interacted PT transcriptome was characterized recently [3].

### Characterization of the pistil-interacted PT transcriptome

The SIV PT bundles from ~2400 pistil explants were used for RNA isolation and to define the *in vivo*-grown PT transcriptome [3]. The *Arabidopsis* SIV PT transcriptome comprises approx. 7000 genes and is ~10% larger than

the mature pollen or *in vitro*-grown PT transcriptome. When compared with pollen grains or 4 h PT, nearly 1500 and 1100 genes respectively are significantly altered in SIV PT. A set of transcripts (~380) is specifically expressed in SIV PT compared with mature pollen grains, 4 h PT and a collection of seven sporophytic tissues [3]. The genes identified in the SIV PT transcriptome represent candidates for the underlying physiological and molecular changes responsible for PT capacitation by the pistil. A systematic characterization, including a reverse genetic analysis described below, will be critical in understanding how this transcriptome is generated and how it is implemented to mediate pollen responses to the complex pistil environment.

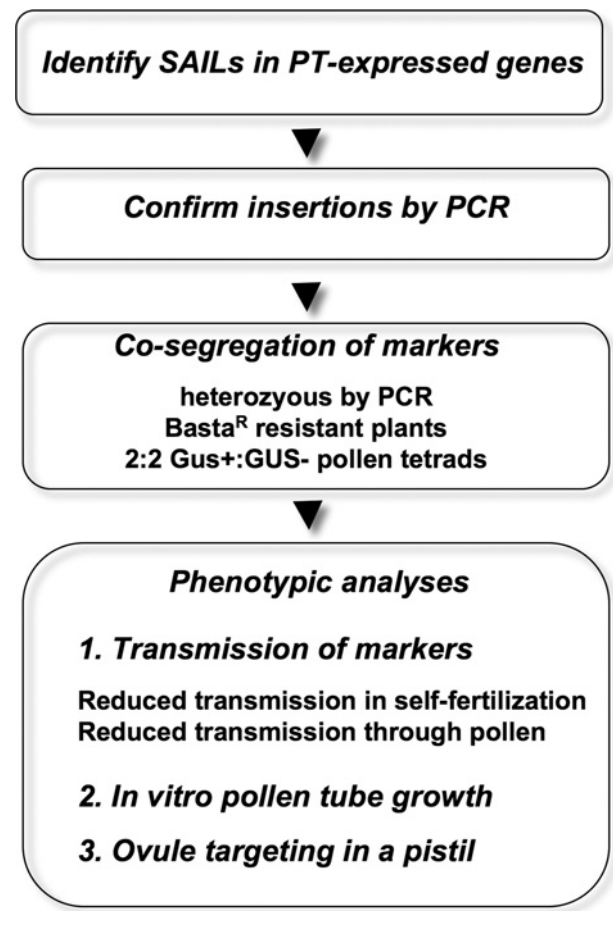
### Blue SAIL lines: valuable tools for genetic analysis of pollen- and PT-expressed genes in *Arabidopsis*

An underlying assumption of microarray analysis of PTs was that genes up-regulated during growth *in vitro* or in SIV PT would be critical for PT growth in the pistil. Analysis of loss-of-function mutants in the genes identified by microarray analysis is one approach to determine whether these genes are functionally significant. In yeast, direct comparison of genes identified by microarray analysis and those identified by functional studies yielded the surprising result that <7% of the genes induced at the mRNA level by a growth condition were required for optimal growth in the same condition [23]. To assess whether microarray analysis of PTs streamlined the process of identifying critical genes, a strategy was developed to analyse a large number of PT mutants (Figure 2). Phenotypic assays were developed to investigate PT behaviour under both *in vitro* and *in vivo* conditions, including late aspects of PT guidance that typically occur deep within a pistil. Furthermore, since pollen mutants are typically maintained as heterozygotes, these assays were designed to distinguish mutant from wild-type pollen produced by heterozygous mutants [3,7].

SAIL (Syngenta *Arabidopsis* Insertion Library) lines are ideal for analysing PT mutant phenotypes and offer several advantages over other mutant populations. Part of the SAIL collection (Blue SAIL lines; numbers beginning 1–456, 1052–1057, 1142–1205 or 1206; [24]) was generated with a T-DNA (transferred DNA) encoding LAT52:GUS (LAT52:β-glucuronidase), a cell-autonomous pollen-specific reporter [24,25]; and a Basta® (a herbicide) resistance gene. T-DNA mutagenesis was carried out on *qrt* plants, which maintain male meiotic products in tetrads [26]. Consequently, Basta®-resistant plants that are heterozygous for a single-locus T-DNA insertion produces tetrads with two mutant pollen grains that stain blue with GUS activity and two wild-type grains that do not stain for GUS activity. GUS expression marks the cytoplasm of PTs grown *in vitro* or in floral tissues, so it is possible to directly compare growth of mutant (blue) PTs with wild-type (unstained) [7,27]. These attributes of the SAIL collection are advantageous for any reverse genetic study because they allow direct analysis of T-DNA copy

## Figure 2 | Overview of reverse genetic analysis of PT-expressed genes

Reverse genetic analysis scheme to characterize the effect of insertions in PT-expressed genes. Individual steps in the process of identifying appropriate insertion lines, or assays used to analyse PT growth and guidance phenotypes, are grouped.



number in the meiotic products of an individual plant and dramatically simplify the process of determining whether a plant is heterozygous (tetrads are two GUS+ to two GUS–, Basta-resistant), homozygous (all four members of tetrads are GUS+, Basta-resistant) or wild-type (all four members of tetrads are GUS–) for a T-DNA-induced mutation.

The Basta-resistance gene in SAIL mutants is important for analysing another aspect of pollen function. When pollen that is heterozygous for a mutation is applied to a wild-type stigma, the frequency that the mutant allele is transmitted to progeny provides a direct and sensitive test for PT function *in vivo* [7]. This assay, which is facilitated by the Basta-resistance gene carried by the T-DNA, yields a value of 0 for genes that are essential for PT growth and 50% Basta-resistant progeny for genes that do not affect pollen function. In summary, Blue SAIL mutants are ideally suited for simultaneously isolating single locus insertion lines in PT-expressed genes and tracking mutant PT behaviours to pinpoint their role in PT growth.

## Transcriptome-assisted reverse genetic analysis of PTs

To determine whether genes that were induced during growth *in vitro* or in the SIV system were functionally significant, a series of sensitive pollen function assays (Figure 2) were used to analyse 50 SAIL lines in 33 genes identified by microarray analysis. Two classes of genes were analysed: (i) those with significantly increased expression in growing 4 h PTs compared with dry pollen; and (ii) those with significantly increased expression in SIV PT compared with 4 h PT [3]. Of the 50 lines analysed, 27 lines showed co-segregation between the two T-DNA markers (LAT52:GUS, Basta-resistance) and a gene-specific PCR-based marker [3], highlighting another useful attribute of SAIL lines: rapid association of PT mutant phenotypes with single-locus-T-DNA insertion.

Of the 27 SAIL lines, insertions in two genes affected PT growth and guidance *in vivo* and insertions in five genes resulted in PT growth defects *in vitro* [3]. Thus seven mutants, out of a starting population of 50 lines, exhibited a detectable phenotype (14%). Previously, a forward genetics screen of 10 000 SAIL lines yielded ~30 mutants defective in pollen and PT function, a yield of 0.3% [7]. Thus performing mutant analysis on a set of PT-induced genes identified by microarray analysis led to a ~45-fold enrichment in the identification of genes with demonstrable function in PTs. So, microarray analysis effectively reduces the size of the genome that must be analysed to identify functionally important genes. However, the majority of genes identified by microarray analysis and tested by reverse genetics had no observable defect. One explanation for this may be functional redundancy, as many microarray-identified genes were members of gene families (see Table S16 in [3]). Systematic crosses can be set up to generate combinations of double mutants which then can be analysed by the genetic scheme described here (Figure 2). This strategy was used recently to identify a role for a pair of PT-expressed receptor-like serine/threonine kinases, ANX1/ANX2, in PT rupture in the ovule [28].

## Future prospects

Protocols developed to characterize pollen [29] and PT [30] proteomes could be applied to the SIV PT system to define the proteins that accumulate in PTs during growth in a pistil. Metabolomics [31] can also be used to determine the inventory of PT small molecules produced in response to growth in the pistil. These studies, coupled with the reverse genetic methods described in the present paper, could facilitate comprehensive analysis of PT capacitation. Another important goal is to identify the signal perception system that allows the PT to respond to PT attractants secreted by ovules [32]. An attractive explanation for PT capacitation is that expression of this perception system is induced during growth in a pistil. Alternatively, capacitation may be mediated by post-translational modifications, not revealed by microarray



analysis, that affect protein abundance and/or modification directly [33].

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