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Comparative Analysis of Biological Models used in the Study of Pollen Tube Growth

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Abstract The mechanisms of pollen tube growth have been studied in a wide variety of plant species. Since the 1990s, with the explosion of molecular genetic analyses in *Arabidopsis thaliana*, most studies started to focus on this model plant. However, because of their particular characteristics, plant species other than *Arabidopsis* are still used to reveal physiological mechanisms and identify novel molecules relating to pollen tube growth, including, for example, lily, tobacco, *Nicotiana alata*, tomato, rice, maize, *Brassica* spp., corn poppy and *Torenia* (Table 1). Here, we designate all of these relatively common experimental plants as "biological models" for the study of pollen tube growth. These models sometimes provide a good first step in the identification of novel physiological mechanisms and molecules. As genome sequencing technologies become more advanced, the difficulty of performing molecular analyses in these biological models will decrease. Thus, a better understanding of these biological models will allow researchers to perform unique studies of pollen tube growth. In this chapter, we compare the characteristics of biological models, focusing on in vitro systems, to facilitate the use of these biological models for in vitro analyses.

1 Comparison of Fundamental Characteristics of Biological Models

Table 2 shows the fundamental characteristics of biological models used in the study of pollen tube growth.

Arabidopsis thaliana is now the most commonly used model plant. This species has many characteristics that are useful in the study of reproductive processes. In addition to the availability of molecular genetic tools for *Arabidopsis*, its small, simple pistil is useful for the observation of entire path of growth of a pollen tube (Hülskamp et al. 1995). In vitro culture of the pollen tube is also possible (Sect. 2). Thus, the molecular mechanisms underlying gametophyte development, pollen–pistil interactions, and pollen tube growth have are also being studied in *Arabidopsis*. The pollen is tricellular (Fig. 1), i.e., it consists of one vegetative cell and two sperm cells, and shows no self-incompatibility. Recent population genomic analyses have shown that *Arabidopsis* became a self-compatible plant about 320 000 years ago because of the selective sweep of a pseudo allele of *SCR* gene, *ΨSCR1* (Shimizu et al.

Table 1 Biological models used in the study of pollen tube growth*

Brassicaceae	Solanaceae	Liliaceae	Poaceae	Papaveraceae	Scrophulariaceae
Arabidopsis thaliana (*Arabidopsis*)	*Nicotiana tabacum* (tobacco)	*Lilium longiflorum* (lily)	*Oryza sativa* (rice)	*Papaver rhoeas* (corn poppy)	*Torenia fournieri* (*Torenia*)
Brassica campestris —	*Nicotiana alata* —	*Agapanthus umbellatus* (*Agapanthus*)	*Zea mays* (maize)		*Antirrhinum majus* (snapdragon)
Brassica oleracea —	*Lycopersicon esculentum* (tomato)				
	Petunia hybrida (petunia)				

* In addition to these commonly used species, various other plants have been used in the study of pollen tube growth, including the following genera (see also Sect. 13.1, below): *Impatiens*, *Portulaca*, *Camellia*, *Tradescantia*, *Gibasis*, *Trifolium*, *Lathyrus*, *Melilotus*, *Pisum*, *Medicago*, *Lotus*, *Prunus*, *Pyrus*, *Ipomoea*, *Secale*, *Gasteria*, *Ornithogalum*, *Helianthus*, *Plumbago* and *Alnus*. Common names are indicated in parentheses below the scientific name; both are used in this chapter

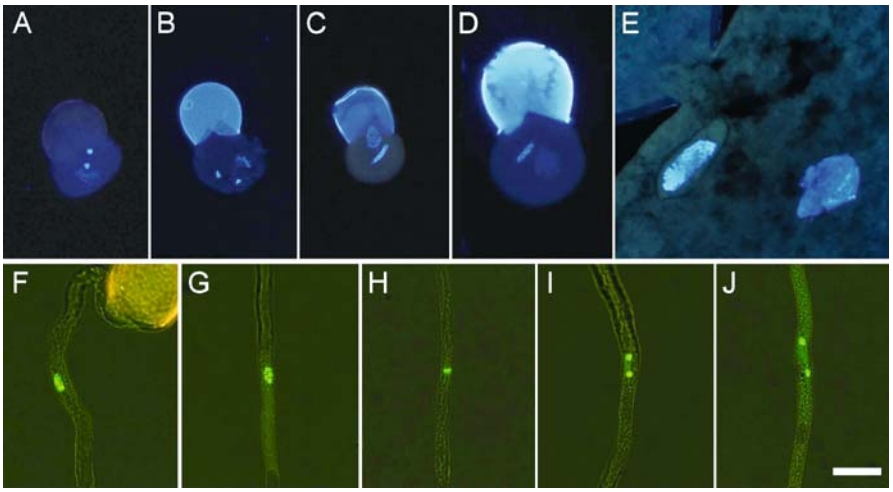

Fig. 1 Pollen grains stained with DAPI (**A** to **E**) and pollen tubes stained with SYTO11 (**F** to **J**). **A** to **E** Pollen grains of *Arabidopsis* (**A**), rice (**B**), tomato (**C**), *Torenia* (**D**) and lily (**E**) were observed after preparation by the squashing method of Sodmergen et al. (1992). Note that all panels are prepared in the same magnification of view. **F** to **J** Pollen tubes of *Torenia* were cultivated in a medium containing 300 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 100 mg/L H_3BO_3, 1% sucrose and 13% PEG 4000 at 30 °C for 4 h, and then they were stained by adding 1000-fold diluted (final) SYTO11 nucleic acid stain (Molecular Probes, Eugene, Oregon, USA). Nuclei of generative and sperm cells at various stages are observed in living pollen tubes. The vegetative nucleus is not observed in panels **F** to **J** but is stained with SYTO11. Bar in **J** = 20 μm for **A** to **J**

2004). It is exciting to speculate that the adaptation of this self-compatible plant, including a reduction in petal size and development of an automatic self-pollination system, occurred in such a short time period, i.e., within 320 000 years (Shimizu et al. 2004). An autogamous plant is useful for genetic analyses, but requires emasculation for artificial and cross pollination. In *Arabidopsis*, fertilization begins 5 h after pollination (Fauré et al. 2002). Each ovary contains about 50 ovules, which are fertilised by each pollen tube from the uppermost to the lowermost ovules (Hülskamp et al. 1995). The growth speed and germination rate of a pollen tube in vitro differ with the plant ecotype; pollen tubes of ecotype Columbia grow faster than those of Landsberg and Wassilewskij (WS).

Tobacco (*Nicotiana tabacum*) produces many pollen grains per anther. Pollen grains germinate with very high frequency (almost 100%) and grow at high rates of speed (~ 200 μm/h) to substantial lengths (over 1 cm; Read et al. 1992). In addition, transient expression in tobacco pollen can easily be induced by bombardment (e.g., Twell et al. 1989; Chen et al. 2002) and stable transformation can be induced using agrobacteria (e.g., Horsch et al. 1985). Thus, tobacco is commonly used for cytological analyses of pollen tube

growth in vitro. Tobacco plants are amphidiploid. The pollen is bicellular, i.e., it consists of one vegetative cell and one generative cell, and shows no self-incompatibility. *Nicotiana alata* has also been used for the study of gametophytic self-incompatibility, including identification S-RNase as a female sporophytic determinant (McClure et al. 1989; Barend et al., this volume). In tobacco, fertilization begins about 40 h after pollination.

Tomato (*Lycopersicon esculentum*) is a commercially important plant that belongs to the family Solanaceae, which also includes tobacco. Genetically modified organisms (GMO) were first commercialised using tomato (flavor saver) in 1994, and a tomato genome project (International Solanaceae Genome Project) launched in 2003. The most commonly used strong promoter for pollen vegetative cells, the *LAT52* promoter, was found in tomato. The pollen protein LAT52 interacts with a receptor-like kinase LePRK2 in what might be an autocrine signaling system (Tang et al. 2002). Tomato is autogamous; the pollen is bicellular (Fig. 1) and shows no self-incompatibility. Fertilization begins about 48 h after pollination.

Lily (*Lilium longiflorum*) has an extraordinarily large genome size compared to *Arabidopsis*. It has been estimated at 34 496 Mbp, i.e., about 300 times that of *Arabidopsis*. Lily cells are also large, reflecting the large genome size (Fig. 1), and, like tobacco, lily produces many pollen grains per anther; therefore, lily is useful for cytological, physiological and biochemical studies of pollen development and pollen tube growth (Hepler et al., this volume). Traditionally, the behaviour of chromosomes during meiosis has been studied in lily (Stern 1985). The isolation of a large number of generative cells at the same developmental stage is also possible, and this has led to the identification of generative-cell-specific proteins and genes (e.g., Ueda and Tanaka 1994; Mori et al. 2003). Additionally, molecules relating to pollen–pistil interactions have been found in lily, using in vitro systems (Kim et al. 2004; Johnson and Lord, this volume). Lily has bicellular pollen, which shows gametophytic self-incompatibility. Fertilization begins about 60 h after pollination occurs by passing through the style tissue, which reaches a length of 10 cm. *Agapanthus umbellatus*, *Hemerocallis* spp., *Ornithogalum* spp., *Gasteria verrucosa* and other *Lilium* spp. all belong to the Liliaceae (although *Gasteria* has also been placed in the Aloaceae) and have often been used to study pollen tube growth (Hepler et al., this volume).

Rice (*Oryza sativa*), one of the most important crops, is the most-consumed crop in the world. The entire genome sequence was the second whole plant genome to be published (International Rice Genome Sequencing Project 2005). Rice pollen is tricellular (Fig. 1). Generally, tricellular pollen is difficult to germinate in vitro (Brewbaker 1967), but pollen tube culture is possible (Kariya 1989; Khatun and Flowers 1995) provided that pollen grains are collected just at flowering (Khatun and Flowers 1995). Thus, rice pollen is rarely used for in vitro studies of pollen tube growth. However, studies of meiosis and anther and ovule development (Itoh et al. 2005) and

genome-wide quantitative trait loci (QTL) analyses of reproductive barriers (Harushima et al. 2001, 2002) have been progressing well. Fertilization begins 30 min after pollination.

Maize (*Zea mays*) another crop with many useful genetic resources available, is tricellular but pollen tube culture is possible (Walden 1993). Maize is suitable for the isolation of sperm cells and female gametophytic cells. Using isolation techniques, in vitro fertilization studies and transcriptome analyses have progressed in maize (Engel et al. 2003; Raghavan 2003; Weterings and Russell 2004). *ZmEA1*, which was found in the cDNA library of the egg cell, is also expressed in the synergid cell and contributes to pollen tube guidance to the female gametophyte (Márton et al. 2005). Fertilization begins 12 h after pollination (Mòl et al. 1994).

Brassica spp. (e.g., *B. campestris* (syn. *rapa*); *B. oleracea*; *B. napus*) and corn poppy (*Papaver rhoeas*) have both been used for studies of self-incompatibility, as have other self-incompatible plants including *Nicotiana alata* (Barend et al., this volume). Sporophytic self-incompatible plants include *Brassica* spp. (Brassicaceae) and sugar potato (*Ipomoea batatas*; Convolvulaceae). In *Brassica*, the female factor was identified as a S-locus receptor kinase, SRK, and the male factor was identified as a small, S-locus cysteine-rich (SCR) basic protein (SP11), which is the ligand of SRK (Takayama and Isogai 2003; Barend et al., this volume). Gametophytic self-incompatible plants include *Nicotiana alata* (Solanaceae), corn poppy (Papaveraceae), *Prunus dulcis* and *Pyrus pyrifolia* (almond and Japanese pear; Rosaceae), *Secale cereale* (rye; Poaceae) and *Antirrhinum* (Scrophulariaceae). In most of them, except for corn poppy and rye, the female factor was identified as S-RNase and the male factor was identified as an S haplotype-specific F-box (SFB) protein, which is related to protein degradation (Kao and Tsukamoto 2004). The pollen of *Brassica* spp. is tricellular, and pollen tube culture is possible (Sect. 2). The pollen of corn poppy is bicellular, and in vitro systems have been used in the study of self-incompatibility (Rudd and Franklin-Tong 2003).

Torenia fournieri possesses a naked embryo sac that protrudes from the micropyle of the ovule. Pollen tube attraction to the embryo sac can be directly observed in this plant (Higashiyama et al. 1998). Laser ablation experiments showed that the source of the attractant is the synergid cell (Higashiyama et al. 2001). The pollen of *Torenia* is bicellular, and the pollen tube grows well in vitro (Fig. 1). Transformation of *Torenia* has been performed for molecular breeding, including modification of flower colour (e.g. Aida et al. 2000). As an experimental plant, *Torenia* has many suitable characteristics for the study of plant reproduction, including the production of large numbers of flowers; relatively short generation time ($\sim$ 3 months from seeds to seeds); low plant height ($\sim$ 40 cm), allowing cultivation in chambers; allogamous reproduction without self-incompatibility, allowing easy control of pollination; and accessibility of the fertilization processes, allowing the observation of the

entire fertilization process (the precise time course of fertilization has also been determined; Higashiyama et al. 1997). Fertilization in *Torenia* begins 9 h after pollination. *Torenia* belongs to the Scrophulariaceae, as do snapdragon and *Mimulus*.

Snapdragon (*Antirrhinum majus*) is a commonly used experimental plant for developmental studies using transposable elements. The pollen of snapdragon is bicellular and the pollen tube grows well in vitro. Mascarenhas and Machlis (1962a) first showed that pollen tubes grow toward calcium ions in an in vitro assay using snapdragon (Johnson and Lord, this volume). The pollen does not show self-incompatibility, although some self-incompatible lines of *Antirrhinum* have been used for the study of gametophytic self-incompatibility (Barend et al., this volume). Fertilization begins about 48 h after pollination (Negre et al. 2003).

Cytoplasmic inheritance (Table 2) is not directly involved in pollen tube growth, but is important when considering the function of pollen in transmitting the paternal genome(s). Maternal, paternal, and biparental inheritance are known in the inheritance of both mitochondria and chloroplasts (plastids) in plants, which differs from the strict maternal inheritance that occurs in animals (Birky 1995). The majority of flowering plants, including biological models listed in Table 2, exhibit maternal inheritance. The inheritance of mitochondria is controlled independently from that of chloroplasts. For example, chloroplasts and mitochondria of *Medicago sativa* (alfalfa) are inherited biparentally and maternally, respectively (Forsthoefel et al. 1992), whereas those of *Musa acuminata* (banana) are inherited maternally and paternally, respectively (Fauré et al. 1994). These manners of cytoplasmic inheritance correlate well with the degradation or amplification of DNA in each organelle of the generative cell (Nagata et al. 1999); in the generative cells of *Medicago sativa*, chloroplast DNA is amplified while mitochondrial DNA is degraded, whereas in *Musa acuminata*, chloroplast DNA is degraded while mitochondrial DNA is amplified.

Plumbago zeylanica and some flowering plants show distinctive characteristics in the distribution of organelle DNAs in the generative and sperm cells (reviewed by Weterings and Russell 2004); these characteristics probably contribute to cytoplasmic inheritance. In *Plumbago zeylanica*, the sperm cell associated with the vegetative nucleus does not contain chloroplasts, but the other sperm cell, which is not associated with the vegetative nucleus, does (Russell 1984). These sperm cells contain amplified DNA (Sodmergen et al. 1995). The former sperm cell preferentially fertilises the central cell, and the latter fertilises the egg cell (Russell 1985). *Plumbago* also has a distinctive female gametophyte, which does not contain a synergid cell, although the base of the egg cell shows characteristic cell walls that resemble the filiform apparatus of the synergid cell (Russell 1985).

In addition to the common biological models mentioned above, there are other existing or developing biological models used in the study of pollen

Table 2 Fundamental characteristics of biological models used in the study of pollen tube growth

	Arabidopsis thaliana	*Brassica campestris*	*Nicotiana tabacum*	*Lycopersicon esculentum*	*Lilium longiflorum*	*Oryza sativa*	*Zea mays*	*Papaver rhoeas*	*Torenia fournieri*	*Antirrhinum majus*
Genome size (Mbp)[1]	125	564	5733	1005	34 496	389–466	2671	2573	—[2]	1568
Chromosome number (*n*)	5	9	24	12	12	12	10	7	9	8
Pollen cell number	Tricellular	Tricellular	Bicellular	Bicellular	Bicellular	Tricellular	Tricellular	Bicellular	Bicellular	Bicellular
Cytoplasmic inheritance	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal	Maternal
Stigma type	Dry	Dry	Wet, lipid-rich	Wet	Wet carbo-hydrate-rich	Dry	Dry	Dry	Dry	Dry
Self-incompatibility	None	Sporophytic	None	None	Gameto-phytic	None	None	Gameto-phytic	None	None
Pollination (in a chamber)	Automatic	—	Automatic	Automatic	—	Automatic	Automatic[3]	—	None[4]	Automatic
Style type	Solid	Solid	Solid	Solid	Hollow	Solid	Solid	Solid[5]	Hollow	Solid
Pollen tube culture	Possible	Possible	Easy	Easy	Easy	Possible	Possible	Easy	Easy	Easy

[1] C-values (http://www.rbgkew.org.uk/cval/homepage.html) except for *Arabidopsis* (*Arabidopsis* Genome Initiative 2000) and rice (Yu et al. 2002; Goff et al. 2002; International Rice Genome Sequencing Project 2005). C-values of *Arabidopsis* and rice are 157 Mbp and 490 Mbp, respectively.
[2] We estimate the genome size of *Torenia* as several hundreds Mbp, judging from the fluorescence intensity of the generative cell nucleus stained with DAPI as shown in Fig. 1.
[3] The frequency of self-pollination is low, because female and male flowers bloom at different positions and times.
[4] Self-pollination never occurs, because of enough stigma-anther separation.
[5] A typical style is absent.

tube growth. For example, genome projects have been launched for *Medicago truncatula* and *Lotus japonicus* (Fabaceae). Interestingly, a relationship between the molecular mechanisms of pollen tube growth and infection thread formation in *L. japonicus* has been implied (Tansengco et al. 2004). *Pisum sativum* (pea), also in the family Fabaceae, is sometimes used for pollen tube microinjection (e.g., Li et al. 1999). *Impatiens*, *Portulaca*, *Camellia*, *Tradescantia* (e.g., spiderwort) and *Gibasis pellucida* (bridal veil) in the Commelinaceae, and *Trifolium* (e.g., clover), *Lathyrus odoratus* (sweet pea), *Melilotus* (e.g., sweet clover) in the Fabaceae, are often used in the classroom because of rapid germination and/or high germination frequency (these plants, except *Portulaca,* all possess bicellular pollen). The pollen of *Impatiens balsamina* (balsam) germinates very quickly (in 1–3 min) and at a high frequency in vitro. The pollen of *Portulaca* also shows a high germination frequency in vitro and is useful for the observation of pollen tubes on the papillar cell of the stigma. The pollen of *Camellia japonica* can germinate at a high rate in vitro, even at cold temperatures ($\sim 5\,^{\circ}C$), although it requires 30 min to several hours to germinate. Various additional plant species have been used for the observation of pollen tubes in the pistil in histochemical, ultrastructural and cytological studies (reviewed by Van Went and Willemse 1984).

In some species of flowering plants, the pollen requires an extraordinarily long time to pass from pollination to fertilization, similar to that of gymnosperms. In *Alnus* (alders), pollination to fertilization takes 2 months; intermittent pollen tube growth occurs, with a clear correlation between pollen tube growth and the developmental stages of the ovule and embryo sac (Sogo and Tobe 2005). These distinctive plant species may provide an opportunity to discover novel, but universal, mechanisms of pollen tube growth.

2
Pollen Tube Growth in Vitro

Upon pollination, the pollen germinates and the pollen tube grows toward the ovule, directed by complex intercellular communication with the pistil. Because the pollen tube growing through the pistil tissue is inaccessible, in vitro systems are used in various biological models. Pollen germination and pollen tube growth can be mimicked in vitro using chemically defined culture media under strictly controlled conditions. In this section, we compare characteristics and conditions of in vitro pollen tube growth in biological models.

2.1
Pollen Type

There are two types of pollen with regard to cell number, i.e., bicellular and tricellular pollen. Pollen mitosis II (division of the generative cell) of bicellu-

lar pollen occurs in the growing pollen tube, whereas that of tricellular pollen occurs in the maturing pollen grain (Fig. 1). The type of pollen is often correlated with the in vitro germination frequency and growth rate; generally, bicellular pollen is more easily cultivated in vitro. As shown in Table 2, bicellular pollen includes that of tobacco, tomato, lily, corn poppy, *Torenia* and snapdragon, and their pollen tubes grow well in vitro. In contrast, *Arabidopsis*, *Brassica* spp., rice and maize have tricellular pollen, and their pollen tubes are relatively difficult to cultivate. Brewbaker (1967) tested about 2000 species of flowering plants and showed that approximately 70% have bicellular pollen and 30% have tricellular pollen. Tricellular pollen was proposed to be a more evolved trait adapted to humid climates; interestingly, all aquatic species with submersed flowers shed pollen in a tricellular stage. Most of these genera are monotypic with respect to cell number, except for *Burmannia*, *Calliandra*, *Comanthosphace*, *Drosera*, *Euphorbia*, *Hymenocrater*, *Ipomoea*, *Lobelia*, *Plantago* and *Ruta*. The genes regulating the timing of division of the generative cell are still unknown, although water uptake may trigger the division of the generative cell in bicellular pollen.

2.2
Biological Models for the Study of Pollen Tube Growth in Vitro

The pollen of *Arabidopsis* and plants of the Solanaceae, including tobacco; Liliaceae, including lily and *Agapanthus*; Scrophulariaceae, including *Torenia*, and corn poppy are often used to study in vitro pollen tube growth. Among these plants, *Nicotiana alata*, corn poppy and self-incompatible *Antirrhinum* lines have been used to study self-incompatibility, and *Torenia* has been used to study pollen tube guidance to the embryo sac. The pollen of *Impatiens*, *Portulaca*, *Camellia*, the Commelinaceae, including spiderwort, and Fabaceae, including clover, is also often used for in vitro studies because of its rapid germination, high germination frequency, length of the pollen tube, and convenience in obtaining flower materials.

2.3
Culture Media and Conditions for Pollen Tube Growth in Vitro

To date, various culture media for in vitro pollen tube culture have been developed; some of these are summarised in Table 3. The medium of Brewbaker and Kwack (1963) is one of the most popular for pollen tube culture. Brewbaker and Kwack (1963) tested the pollen of several hundred species, particularly that of petunia and *Ornithogalum virens* (Liliaceae), to determine a basal medium (Table 3). The media of Hodgkin (1983), Jahnen et al. (1989) and Read et al. (1993) are also popular and have been used as bases from which to develop novel media for biological models. Variations in the media used for *Arabidopsis* pollen still exist, but the media used for lily

Table 3 Various media used for pollen tube culture*1

	petunia and *Ornithogalum* (Liliaceae)	*Arabidopsis*			tobacco	
Original paper	Brewbaker and Kwack 1963	Hodgkin 1983*2	Li et al. 1999	Derksen et al. 2002	Read et al. 1993	Chen et al. 2002
NH_4NO_3	—	—	—	—	—	—
HNO_3	—	—	—	—	—	100
KNO_3	100	100 (0.99 mM)	—	—	—	—
KCl	—	—	—	—	75 (1.0 mM)	—
$Ca(NO_3)_2 \cdot 4H_2O$	300	600 (2.54 mM $Ca(NO_3)_2$)	236 (1 mM)	—	—	—
$CaCl_2$	—	—	111 (1 mM)	528 (700; $CaCl_2 \cdot 2H_2O$)	111 (1.0 mM)	700
$MgSO_4 \cdot 7H_2O$	200	217 (0.88 mM)	—	—	96 (0.8 mM $MgSO_4$)	409 (200; $MgSO_4$)
$Mg(NO_3)_2 \cdot 6H_2O$	—	—	—	—	—	—
$MgCl_2$	—	—	—	—	—	—
KH_2PO_4	—	—	—	—	—	—
$K_3PO_4 \cdot H_2O$	—	—	—	—	—	—
$MnSO_4 \cdot 4H_2O$	—	—	169 (1 mM)	—	—	—
$ZnSO_4 \cdot 7H_2O$	—	—	—	—	—	—
H_3BO_3	100	100 (1.62 mM)	100	100	99.2 (1.6 mM)	100
$CuSO_4 \cdot 5H_2O$	—	—	—	—	(30 μM $CuSO_4$)	—
$Na_2MoO_4 \cdot 2H_2O$	—	—	—	—	—	—
Sucrose	10%	20% (585 mM)	18%	20%	5%	2%
Polyethylene-glycol (MW)	—	—	—	3% (4000)	12.5% (6000)	15% (3350)
Casein	—	—	—	—	300	—
MES	—	—	—	—	15 mM	20 mM
TAPS	—	20 mM	—	—	—	—
pH	—	8 (NaOH)	7	—	5.9 (KOH)	6
Agarose	—	0.5%	—	0.7% (Bacto-Agar)	—	—
Rifampicin	—	—	—	—	10	—

pollen have been improved over many years and have almost converged (Table 3).

At least three substances, plus water, are required in the pollen tube culture medium. The first substance is calcium, which is necessary for pollen tip growth (see Hepler et al., this volume). Calcium is usually added to the medium at 10–600 mg/L as nitrate or chloride salts. The second substance is borate, which may be necessary for cell wall formation (Loomis and Durst 1992). Borate is usually added as boric acid, and a concentration of 100 mg/L

Table 3 (continued)

	Nicotiana alata				*Agapanthus*	corn poppy	*Torenia*
Original paper	Jahnen et al. 1989	Holdaway-Clarke et al. 2003[*3]	Kim et al. 2003	Prado et al. 2004	Malhó and Trewavas 1996	Franklin-Tong et al. 1988	Higashi-yama et al. 1998[*4]
NH_4NO_3	—	—	—	—	—	—	80
HNO_3	—	—	—	—	—	—	—
KNO_3	100	—	100 (0.99 mM)	—	—	—	125
KCl	—	7.5 (0.1 mM)	—	75 (1.0 mM)	200	—	—
$Ca(NO_3)_2 \cdot 4H_2O$	700	—	300 (1.27 mM)	—	—	—	500
$CaCl_2$	—	11.1 (0.1 mM)	—	55.5 (0.5 mM)	200	272 (360; $CaCl_2 \cdot 2H_2O$)	—
$MgSO_4 \cdot 7H_2O$	200	—	—	—	—	—	125
$Mg(NO_3)_2 \cdot 6H_2O$	—	—	—	—	—	100	—
$MgCl_2$	—	—	—	—	200	—	—
KH_2PO_4	—	—	—	—	—	—	125
$K_3PO_4 \cdot H_2O$	—	—	—	—	—	100	—
$MnSO_4 \cdot 4H_2O$	—	—	—	—	—	—	3
$ZnSO_4 \cdot 7H_2O$	—	—	—	—	—	—	0.5
H_3BO_3	100	99.2 (1.6 mM)	10 (0.162 mM)	99.2 (1.6 mM)	100	100	10
$CuSO_4 \cdot 5H_2O$	—	—	—	—	—	—	0.025
$Na_2MoO_4 \cdot 2H_2O$	—	—	—	—	—	—	0.025
Sucrose	2%	7%	10%	6%	2.5%	12%	1%
Polyethylene glycol (MW)	15% (4000)	—	—		—	—	13% (4000)
Casein	—	—	—	—	—	—	500
MES	—	15 mM	—	0.05 mM	—	—	—
TAPS	—	—	—	—	—	—	—
pH	—	6 (KOH)	5.2	6.0	6.0	—	5.8 (without adjustment)
Agarose	—	—	1.0%	—	—	0.4%	1.5%[*5]
Rifampicin	—	—	—	—	—	—	—

[*1] Units are mg/L unless otherwise noted.
[*2] Originally established for pollen tube culture in *Brassica oleracea* but often used for pollen tube culture in *Arabidopsis*.
[*3] Established for pollen tube culture in *Lilium formosanum*.
[*4] The 4% sucrose is now replaced by 13% PEG 4000 to improve the fertilization frequency as described in Sect. 13.2.
[*5] Ultra-low gelling temperature agarose.

appears sufficient for most flowering plants. The third substance is sucrose, which is necessary to adjust the osmotic pressure and may be used as a carbon source for respiration and the synthesis of starch, lipids, amino acids

and nucleic acids (Vasil 1987). Sucrose is the most suitable sugar source in most flowering plants (Vasil 1987). The optimum osmotic pressure differs among biological models; for example, the optimum sucrose concentration is 10–20% for *Arabidopsis* (Hodgkin 1983; Li et al. 1999; Derksen et al. 2002; Schreiber and Dresselhaus 2003), 9–18% for tobacco (Cheung et al. 2002; Romagnoli et al. 2003), 5–10% for lily (Vidali et al. 2001; Holdaway-Clarke et al. 2003; Kim et al. 2003; Prado et al. 2004), 2.5% for *Agapanthus* (Malhó and Trewavas 1996), 11% for maize (Walden 1993), 20% for rice (Kariya 1989), 12% for corn poppy (Franklin-Tong et al. 1988) and 5% for *Torenia* (Higashiyama et al. 1998).

In addition, other inorganic ions (e.g., K^+, Mg^{2+}; Brewbaker and Kwack 1963), buffers to control pH (e.g., 2-Morpholinoethanesulfonic acid, monohydrate (MES); Tupy and Rihova 1984) and organic compounds (e.g., amino acids, casein hydrolysate; reviewed by Vasil 1987) are used in the media to promote pollen tube germination and growth, depending on the plant species. The frequency and timing of the generative-nucleus division also depended on the chemical composition of the medium in tobacco (Read et al. 1993). It is noteworthy that polyethyleneglycol (PEG) 4000–8000 has a dramatic effect on pollen tube germination and growth (Zhang and Croes 1982; Jahnen 1989; Read et al. 1993; Barinova et al. 2002), although the physiological action of PEG is unknown. PEG has been widely used in pollen tube culture media for *Arabidopsis*, tobacco, *Torenia* and snapdragon (Barinova et al. 2002; Schreiber and Dresselhaus 2003; Table 3). In *Torenia*, PEG 4000 increased the viability of both the pollen tube and the naked embryo sac, whereby the frequency of pollen tube attraction in vitro increased fourfold (Higashiyama et al. 2000). However, high concentrations of PEG tend to precipitate in solid media. Thus PEG is sometimes replaced by sucrose when used in solid media (e.g., Cheung et al. 2002). Osmotic potential of 15% PEG 4000 (327 mOsm/kg) corresponds to that of 9% sucrose (325 mOsm/kg) (Jahnen et al. 1989). In the medium for *Torenia*, 13% PEG 4000 in 1.5% ultra-low gelling temperature agarose is the maximum concentration that balances the effect of PEG 4000 and the extent of precipitation (Table 3). As in cell fusion experiments, PEG appears to remain effective for only one month after its dissolution in water, and its effect is also lost by autoclaving.

The effectiveness of simple media should be tested first. Environmental conditions, such as humidity and aeration, and physiological conditions of the flower are critical and can be tested using a simple medium. Pollen germination may also depend on the population effect, i.e., the pollen concentration. Phytosulphokine (PSK), a peptide hormone of flowering plants, is an intercellular signalling molecule (Chen et al. 2000). It should also be noted that Na^+ strongly inhibits pollen tube growth in most flowering plants. Thus, KOH, rather than NaOH, is recommended to adjust the pH. The optimal temperature for pollen tube culture is usually 20–30 °C, and the growth rate of pollen tubes depends on the temperature.

The medium for the in vitro *Torenia* system (Higashiyama et al. 1998, 2001) is described in Table 3. *Torenia* pollen tubes grow well in a simple medium containing 300 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 100 mg/L H_3BO_3, 1% sucrose and 13% PEG 4000 (Fig. 1), but this medium does not support the culture of ovules that have a naked embryo sac. Therefore, Nitsch's medium (1951), originally established for the culture of excised ovaries, was modified for the cultivation of both the pollen tube and ovule together.

2.4 Semi-in Vitro Pollen Tube Growth

A pollen tube can grow autonomously to some extent using its own nutrients; however, the resultant pollen tube is much shorter than the style. Pollen tubes perceive many molecules in the extra-cellular matrix (ECM) of the pistil, and no medium can produce pollen tube growth similar to that which occurs in the pistil. Thus, a semi-in vitro (also called semi-in vivo) system is sometimes used for pollen tube culture, wherein pollen tubes grow through a cut style. Pollen tubes germinate on the stigma, grow through the cut style, and enter the culture medium from the cut end of the style. Pollen tubes grown semi-in vitro show a higher growth rate and more normal morphology than those germinated on artificial medium. For example, in *Torenia* at 25 °C, pollen tubes grow at 2.3 mm/h in the pistil (in vivo), 0.6 mm/h in medium after germinating on the medium (in vitro), and 1.2 mm/h in the same medium after germinating on the stigma and passing through the style (semi-in vitro). Moreover, semi-in vitro growth is necessary for the capacitation-like mechanism of the pollen tube to respond to attractant from the synergid cell in *Torenia* (Higashiyama et al. 1998). Similar phenomena have been observed in lily (Janson 1993) and *Aechmea fasciata* (Bromeliaceae; Vervaeke et al. 2003); pollen tubes grown semi-in vitro more frequently penetrate the micropyle of the ovule than those grown in vitro. Among the biological models, semi-in vitro pollen tube growth has also been used in tobacco (Cheung et al. 1995), as described in (Johnson and Lord, this volume).

For semi-in vitro pollen tube growth, the medium must be prepared as carefully as that for in vitro pollen tube culture. It is important that the style tissue, including the cut end, is well-maintained on the medium so that pollen tube growth inside the tissue is supported. Any self-incompatibility of the plant species should also be noted before semi-in vitro culture is performed.

3 Study of Pollen Tube Guidance in Vitro

During growth in the pistil, the pollen tube perceives multi-step directional controls from the pistil (see Johnson and Lord, this volume). To study the

mechanisms of pollen tube guidance and to identify guidance cues, many in vitro systems have been developed using various biological models. In this section, we compare such in vitro systems to illustrate how the characteristics of each biological model have been used to study pollen tube guidance.

3.1 In Vitro Systems to Identify Candidate Chemoattractants

Many in vitro systems have been developed to evaluate pollen tube chemotropism; some of these are summarised in Fig. 2. According to Mascarenhas and Machlis (1962b), Van Tieghem (1869) was the first to suggest the possibility of chemo-attraction of the pollen tube in vitro. He cultivated ovules with pollen tubes for some species of flowering plants and observed that the pollen tube grew toward the ovule. Since then, many plant biologists have attempted to identify the guidance cue that navigates the directional growth of the pollen tube with various in vitro tests, using tissues of the pistil (Mascarenhas and Machlis 1962b). Biochemical properties, such as heat-stability and molecular size, were examined in various plants using these in vitro systems until the 1960s. These attempts led to the screening of inorganic ions. Mascarenhas and Machlis (1962a) identified calcium ion derived from the pistil tissue as a candidate chemoattractant of the pollen tube using an in vitro system in snapdragon, as shown in Fig. 2A. Snapdragon was chosen because it consistently showed chemotropic responses in various in vitro sys-

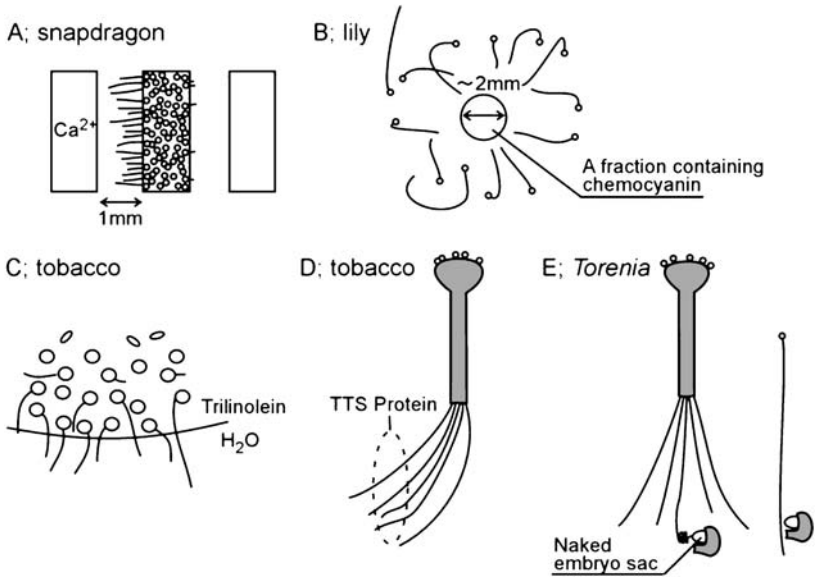

Fig. 2 Various in vitro systems used to assay pollen tube chemotropism

tems and because large numbers of flowers could be obtained throughout the year (Mascarenhas and Machlis 1962c).

A number of histochemical analyses have supported these results by showing the existence of abundant calcium ion along the path of the pollen tube, especially in the synergid cell (reviewed by Higashiyama 2002). However, it was difficult to distinguish between guidance and growth stimulation using classical in vitro tests. In addition, pollen tubes tend to grow parallel when pollen grains are placed in a row. There was no evidence that calcium ion was the true attractant in the pistil; therefore, the existence of a pollen tube attractant has been a confounded issue. For example, Heslop-Harrison and Heslop-Harrison (1986) discussed the existence of the attractant in their review entitled "Pollen-tube chemotropism: fact or delusion?" Kim et al. (2004) finally identified a plantacyanin (blue-copper cell wall protein), a 9.9-kDa basic protein named chemocyanin, in the stigma extract of lily (Johnson and Lord, this volume). The function of plantacyanins in plant cell wall is unknown, but many are capable of redox reactions. In their assays, chemocyanin showed chemoattraction of the pollen tube (e.g., Fig. 2B). Lily is one of the species that shows typical chemotropic responses in vitro to pistil tissues, including the stigma and the ovule (Miki 1954; Welk et al. 1965). Because of the large pollen size, it is also possible to place individual pollen grains in a row to clearly observe the behaviour of each pollen tube (Fig. 2B).

Two types of stigma are recognised: wet and dry (Heslop-Harrison and Shivanna 1977; Table 2). The stigma of lily is wet, with a carbohydrate-rich exudate. In contrast, the stigmas of tobacco and petunia are wet, but lipid-rich. In other plants, such as *Arabidopsis*, *Brassica* spp., rice, maize, corn poppy, *Torenia* and snapdragon, the stigma is dry. The lipid fraction of the stigma exudate contains many triacylglycerides; one of these, trilinolein (unsaturated triacylglyceride), allows pollen tubes to penetrate stigmas that have had genetic ablation of the secretory zone and cannot produce exudate. In an in vitro assay, pollen grains placed in trilinolein germinated in the vicinity of water solidified with 0.7% agarose, and some pollen tubes nearest to the trilinolein–water boundary grew toward the water (Fig. 2C; Wolters-Arts et al. 1998). Few pollen grains placed in other saturated triacylglycerides germinated, and those that did showed no directional growth. A difference in water supply was also evident when different lipids were used. Thus, adequate water supply, depending on physicochemical properties of the lipids, seems necessary for pollen tube germination and directional growth (Wolters-Arts et al. 1998). Similar results were obtained using an in vitro system of *Nicotiana alata*; these results supported the conclusion that the gradient of water should be the guidance cue (Lush et al. 1998). The *Arabidopsis* mutant *pollen–pistil interaction (pop)1* is defective in the synthesis of long-chain lipids, and its pollen does not become hydrated on the stigma because of an impaired pollen coat (Preuss et al. 1993). Application of trilinolein to the stigma enabled *pop1*

pollen grains to produce tubes that penetrated the stigma (Wolters-Arts et al. 1998). Thus, trilinolein and an adequate water gradient are likely to play essential roles, even in dry stigmas.

The style is the tissue that connects the stigma and the ovary. The length of the style differs among plant species: for example, the style measures ~ 100 μm in *Arabidopsis*, ~ 2 mm in rice, ~ 2 cm in *Torenia*, ~ 4 cm in tobacco, ~ 10 cm in lily and ~ 30 cm in maize. Two main types of style are recognised, i.e., "hollow" (open; e.g., lily and *Torenia*) and "solid" (filled with transmitting tissue; e.g., *Arabidopsis*, *Brassica* spp., tobacco, tomato, petunia, rice, maize, corn poppy and snapdragon). In the style, pollen tubes generally grow straight toward the ovary at high growth rates. Cheung et al. (1995) used an in vitro system to test whether transmitting-tissue-specific (TTS) proteins of tobacco have the ability to attract the pollen tube (Fig. 2D). TTS proteins are arabinogalactan proteins (AGPs) in the ECM of the transmitting tissue (style) that are incorporated into the cell wall of the pollen tube and promote tube growth. The authors used pollen tubes growing semi-in vitro to examine changes in the direction of growth of the pollen tube, and observed that pollen tubes turned toward the medium containing TTS proteins (Fig. 2D). It has been argued whether this behaviour of pollen tubes actually indicates the ability of TTS proteins to attract the pollen tube, because it appeared difficult to distinguish between growth promotion and attraction in this system (Lush 1999). However, growth promotion simply cannot account for the directional change of the pollen tube. Other molecules that promote pollen tube growth, such as sucrose, do not show the same effect (Cheung et al. 1995). Interestingly, Mascarenhas and Machlis (1962a) similarly observed that the effect of calcium ion was apparently different from that of sucrose and yeast extract. In contrast, Lush (1999) pointed out that the pollen tubes in the in vitro system of Cheung et al. (1995) did not show trapped behaviour at the point where the concentration of TTS proteins was maximal. In fact, such a trapped behaviour has not been demonstrated well in vitro, except for the in vitro *Torenia* system, as described below. In the developing nervous system, milestones that emit the attractant protein, netrin, exist along the path of the growth cone. The high concentration of netrin renders the growth cone non-sensitive to netrin, causing the cone to approach the next milestone (Shirasaki et al. 1998). Although it is unknown whether the reproductive system has a similar mechanism, these factors make it difficult to demonstrate pollen tube attraction in vitro in a convincing manner.

In the ovary, pollen tubes change their behaviour from straight to meandering growth, climb up the funiculus of the ovule to enter the micropyle, and then grow toward the target female gametophyte, the embryo sac. The entrance of the micropyle seems the most plausible site for the release of a chemoattractant. In *Arabidopsis* mutants defective in embryo sac development, the embryo sac was shown to be necessary for directional pollen tube growth toward the funiculus and the micropyle (Hülskamp et al. 1995; Ray

et al. 1997; Shimizu and Okada 2000). However, the nature of the guidance cue remains unknown. Higashiyama et al. (1998) developed an in vitro system in *Torenia* whereby pollen tubes growing semi-in vitro through the cut style grew toward the micropylar end of the naked embryo sac in the medium, with no need to contact surrounding sporophytic tissues (Fig. 2E). In most cases, pollen tubes did not enter the embryo sac smoothly; rather, they continued to grow toward the micropylar end of the embryo sac, but slipped on the surface of the filiform apparatus of the synergid cell and formed narrow coils (Fig. 2E). Moreover, when an ovule that had attracted, but not received the pollen tube, was moved using a micromanipulator, the pollen tube was observed to trail the embryo sac (unpublished data). This pollen tube behaviour clearly indicates that some diffusible signal is derived from the micropylar end of the embryo sac.

Laser ablation experiments in the in vitro *Torenia* system have shown that the two synergid cells were the source of the attractant (Higashiyama et al. 2001). In *Arabidopsis*, a reverse genetic study of the synergid cell-specific MYB 98 transcription factor confirmed that the synergid cell governs pollen tube guidance at the entrance of the micropyle (Kasahara et al. 2005). Strong species specificity is observed in the pollen tube attractant, suggesting that the attractant may be some molecule, such as a peptide, synthesised in the synergid cell, rather than calcium ion or GABA (Palanivelu et al. 2003; Higashiyama et al. 2003). The attractant chemical is still unknown, although ZmEA1, which is expressed in the egg apparatus of maize, is one of the candidates. ZmEA1 is secreted near the micropyle, as observed using a green fluorescent protein (GFP) fused with ZmEA1, and governs pollen tube guidance at the micropyle, as observed in RNAi knockdown lines (Márton et al. 2005). Proteins similar to ZmEA1 exist only in monocots (McCormick and Yang 2005), and the ability of ZmEA1 to attract pollen tubes has not been shown.

3.2 Comparison of Biological Models with Regard to in Vivo Pollen Tube Guidance

The architecture of the pistil and ovary (including its size and the number of ovules) differs among biological models. As described above, there are different types of stigma and style, and their sizes also differ considerably. For example, there are several types of placenta (placentation) such as parietal (e.g., *Arabidopsis*, *Brassica* spp. and corn poppy), axile (e.g., tobacco, tomato, petunia, lily, *Torenia* and snapdragon) and basal (e.g., rice and maize). In *Arabidopsis*, a typical placenta is not observed, and the placental tissue is usually called a septum. The number of ovules in one ovary is $\sim$ 50 in *Arabidopsis*, $\sim$ 3000 in tobacco, $\sim$ 400 in lily, 1 in rice, and $\sim$ 500 in *Torenia*.

The size of the ovule may affect the number of steps involved in pollen tube guidance by the embryo sac because the effective distance of chemoattraction

is limited mathematically (Lush 1999). In the in vitro *Torenia* system and *Arabidopsis*, the effective distance of attraction by the synergid cell is only a few hundred micrometers at maximum (Higashiyama et al. 2003; Kasahara et al. 2005). In *Arabidopsis*, pollen tube guidance at the ovule is governed by the embryo sac, with at least two control steps (Shimizu and Okada 2000) but in larger ovules more steps may be necessary.

4 Perspectives

Here, we have compared the characteristics of biological models used in the study of pollen tube growth, with a special focus on in vitro culture. Use of these biological models will provide opportunities to identify novel genes, molecules and physiological mechanisms involved in pollen tube growth and check the universality and specificity of each finding. It is foreseen that a large amount of data collected in the near future will focus on *Arabidopsis*. However, the differences outlined in this chapter stress that extrapolations to other species must be critically examined using in vitro systems of other biological models.

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