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Pollen Tube Growth and Guidance Is Regulated by *POP2*, an *Arabidopsis* Gene that Controls GABA Levels

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Summary

During angiosperm reproduction, pollen grains form a tube that navigates through female tissues to the micropyle, delivering sperm to the egg; the signals that mediate this process are poorly understood. Here, we describe a role for γ-amino butyric acid (GABA) in pollen tube growth and guidance. In vitro, GABA stimulates pollen tube growth, although vast excesses are inhibitory. The *Arabidopsis POP2* gene encodes a transaminase that degrades GABA and contributes to the formation of a gradient leading up to the micropyle. *pop2* flowers accumulate GABA, and the growth of many *pop2* pollen tubes is arrested, consistent with their in vitro GABA hypersensitivity. Some *pop2* tubes continue to grow toward ovules, yet they are misguided, presumably because they target ectopic GABA on the ovule surface. Interestingly, wild-type tubes exhibit normal growth and guidance in *pop2* pistils, perhaps by degrading excess GABA and sharpening the gradient leading to the micropyle.

Introduction

GABA is a four-carbon ω-amino acid that functions in metabolism and cell signaling from prokaryotes to animals. In vertebrates, it is an inhibitory neurotransmitter, an inhibitor of cortical neuronal cell divisions, a paracrine signal, and a growth factor (Pinal and Tobin, 1998). In microbes, it serves as a nitrogen and/or carbon source, and in higher plants, it has been implicated in pH regulation, nitrogen storage, development, and pathogen defense (Shelp et al., 1999). Here, we demonstrate that disrupting GABA degradation in *Arabidopsis* flowers causes aberrant pollen tube growth and infertility. Our results define a role for GABA in plant reproduction.

In flowering plants, pollination begins when haploid pollen grains (male gametophytes) are deposited on the diploid female pistil (Figure 1A). The pollen germinates and forms a pollen tube—a polar process that transports the cellular contents, including two sperm, to its elongating tip. The pollen tube migrates past several different cell types before arriving at an ovule where it targets the haploid egg, one of the seven cells of the embryo sac (female gametophyte). In many species, a single tube is guided toward each ovule micropyle (Lord, 2000; Figure 1B). An intracellular calcium gradient, dependent on the activity of RhoGTPase, is required for growth polarity (Li et al., 1999).

Several extracellular pollen growth and guidance signals have been described (Johnson and Preuss, 2002). Lipids in the stigma exudate and pollen coat mediate pollen hydration (Preuss et al., 1993; Wolters-Arts et al., 1998). Rapidly evolving pollen coat proteins also affect hydration, perhaps defining species identity (Mayfield et al., 2001). In the transmitting tract, arabinogalactans such as the *Nicotiana* TTS1 and TTS2 proteins are secreted into the extracellular matrix and provide nutritional and guidance cues that support tube migration (Wu et al., 2000). Adhesion of pollen tubes to the transmitting tract is regulated in lily by pectin and a 9 kd lipid transfer protein (Mollet et al., 2000). Signals that mediate later events, including guidance and targeting to the micropyle, remain largely undefined.

Genetic screens have identified mutants defective in late stages of guidance; while some of these mutations result in abnormal ovule or embryo sac development (reviewed in Palanivelu and Preuss, 2000), others retain structural integrity and are morphologically normal. Both the haploid embryo sac and diploid ovule tissues are required to direct pollen tubes to the micropyle. The *Arabidopsis ino* mutant confirms a role for diploid ovule cells (Villanueva et al., 1999). Other mutants point to an additional role for haploid gametophytes: *Arabidopsis* mutants with severe embryo sac defects do not promote pollen tube guidance, despite the presence of apparently normal diploid ovule tissue (Ray et al., 1997; Shimizu and Okada, 2000). Ablation studies implicate haploid synergid cells as a likely source for these short-range signals (Higashiyama et al., 2001). Once gaining entry to the ovule, pollen tube growth is arrested in a synergid cell, a process termed pollen tube reception (Huck et al., 2003). Signals from the embryo sac regulate tube reception; mutations in GFA2 (Christensen et al., 2002), *sirene* (Rotman et al., 2003), and *feronia* (Huck et al., 2003) disrupt these signals and cause sterility, despite pollen tube arrival at the embryo sac.

The recessive *pop2* mutation has a unique phenotype, affecting the behavior of male and female tissues; even so, *pop2* females are fertile when crossed to wild-type, indicating that the egg and other embryo sac cells are functional (Wilhelmi and Preuss, 1996). *pop2* pollen tubes grow through the mutant and wild-type transmitting tracts at a normal rate, but only in mutant pistils do they show late-stage defects, failing to adhere to ovule cells and bypassing the micropyle (Wilhelmi and Preuss, 1996). This mutation consequently provides an opportunity to dissect the interactions between haploid pollen tubes and diploid ovules. Here, we identify the *Arabidopsis POP2* gene as a GABA transaminase and demonstrate that decreased POP2 activity leads to increased levels of GABA throughout the ovule, coupled with aberrant growth and guidance of *pop2* pollen tubes.

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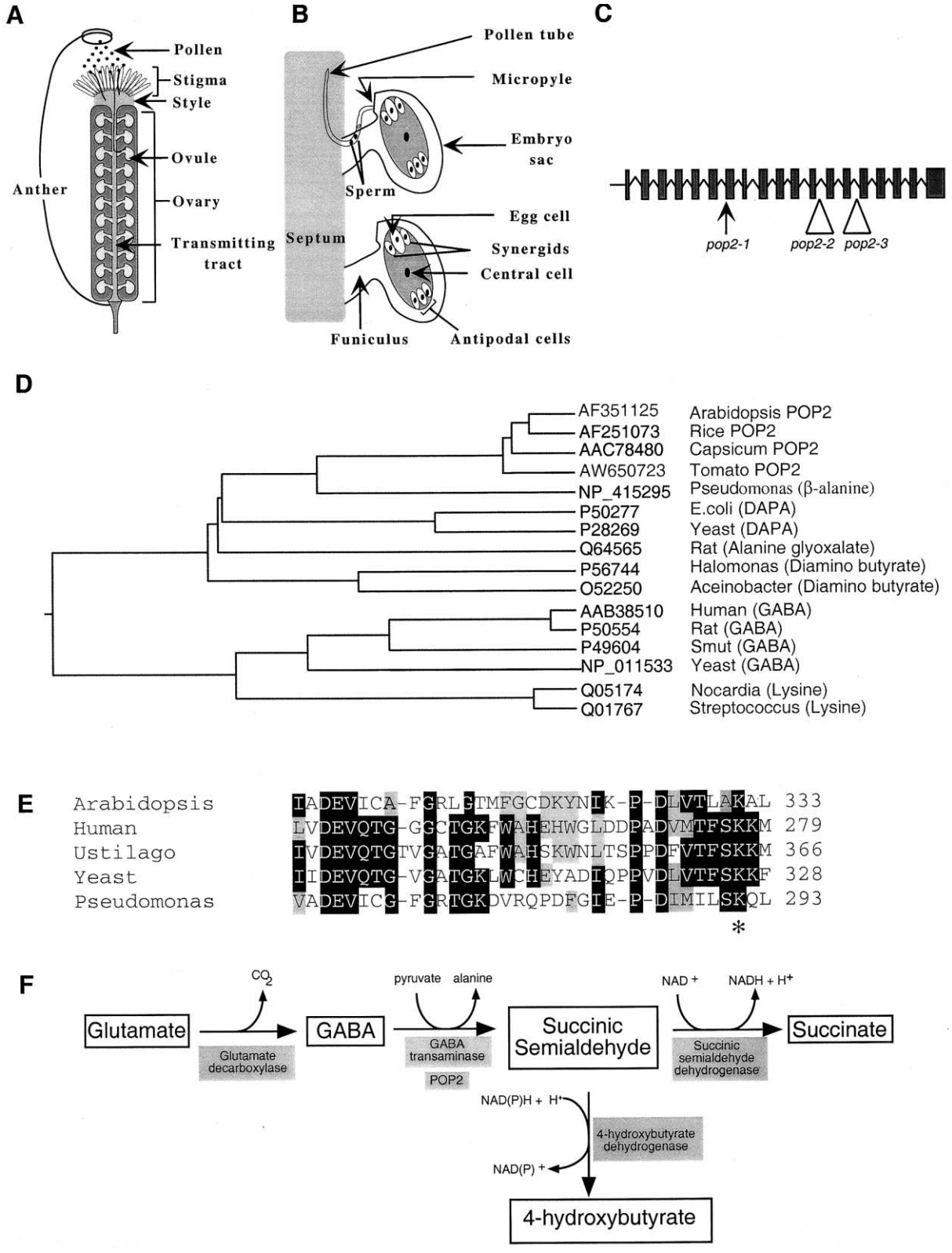


Figure 1. Pollination Requires *POP2*, a GABA Transaminase

(A) Male and female structures of an *Arabidopsis* flower. Pollen from the anther lands on the stigma where it hydrates, germinates, and extends a tube through the style to reach the transmitting tract. The tube emerges out of the tract to target the ovule; several ovules reside within the ovary.

(B) Final stages of pollen tube growth. When the pollen tube emerges from the transmitting tract, it reaches the septum, where the

Table 1. Abundance of Compounds in Flowers (μmol/g Wet Weight)

Major Amino Acids[a]	*POP2*[b]	*pop2-1*[b]	Fold Difference[c]
GABA	0.20 ± 0.06	22 ± 0.45	113
β-alanine	0.16 ± 0.04	0.88 ± 0.17	5.7
Alanine	1.4 ± 0.45	1.6 ± 0.79	1.1
Asparagine	1.6 ± 0.76	2.4 ± 1.9	1.5
Aspartic acid	2.4 ± 0.66	1.4 ± 0.32	0.60
Glycine	2.1 ± 0.45	3.8 ± 0.74	1.8
Glutamate	3.4 ± 0.73	2.6 ± 0.60	0.74
Glutamine	2.5 ± 1.14	6.8 ± 6.1	2.2
Proline	4.9 ± 1.1	2.9 ± 0.83	0.58
Serine	2.5 ± 0.82	2.1 ± 0.66	0.86
Threonine	1.7 ± 0.40	1.3 ± 0.34	0.80
Valine	0.66 ± 0.07	0.69 ± 0.11	1.1
GABA Shunt Components			
Succinic semialdehyde	<0.01	<0.01	NA[d]
Succinic acid	0.068 ± 0.03	0.064 ± 0.01	0.94
4-hydroxybutyrate	1.6 ± 0.81	2.3 ± 0.45	1.4

[a] Other amino acids, including arginine, ethanolamine, histidine, isoleucine, leucine, lysine, methioine, L-methyl histidine, phenylalanine, phosphoserine, tyrosine, and tryptophan showed less than 2-fold variance between *POP2* and *pop2-1*.
[b] Values represent the mean of three independent experiments (±standard error).
[c] Student's T-test comparing *pop2-1* and wild-type showed a significant difference for GABA and β-alanine ($p < 0.0004$ and $p < 0.005$, respectively).
[d] NA , not applicable.

Results

Identifying the *POP2* Gene

We mapped *pop2-1* to a 25 kb region on chromosome 3 (BAC T6O1) containing four predicted genes. Expression of these candidates was assessed by RT-PCR of wild-type and mutant flower mRNA; only one (*POP2*) exhibited significantly reduced expression in mutants (data not shown). We confirmed the predicted gene structure by amplifying and sequencing the wild-type *POP2* cDNA. Sequencing *pop2-1* genomic DNA revealed a G→A transition in the 3′ splice site acceptor for exon 7 (Figure 1C). We used a *pop2-1* dCAPS marker (Neff et al., 1998) to show complete linkage of this transition to the mutant phenotype in 517 sterile progeny from a *pop2-1*/+ parent. Previously, a second locus (*pop3-1*) also cosegregated with sterility (Wilhelmi and Preuss, 1996); subsequent analyses demonstrated that the requirement for *pop3-1* correlated with an embryonic lethal (*emb*) defect tightly linked to *pop2-1* and not *pop2-1* itself. In the absence of the *emb* defect, *pop2-1* is sufficient to cause defects in pollen tube growth, guidance, and fertility. A wild-type copy of *POP2*, introduced into *pop2-1*/+ plants, fully restored the wild-type phenotype to *pop2-1*/*pop2-1* homozygous progeny, confirming the identity of the gene.

POP2 Encodes a GABA Transaminase

POP2 is a single copy *Arabidopsis* gene; closely related genes are present in rice, *Capsicum*, and tomato (Figure 1D). The predicted protein is a class III transaminase (PFam E value, 9e-87) with a conserved binding site for a pyridoxal phosphate cofactor (Figure 1E) and a predicted mitochondrial targeting signal. To identify the POP2 substrate, we analyzed the amino acid composition of mutant and wild-type flower extracts. In mutant flowers, GABA was elevated 100-fold, while concentrations of nearly every other amino acid remained unchanged (Table 1). The sole exception was a 5-fold enrichment in β-alanine; thus, while GABA is the primary substrate, POP2, like many transaminases, can interact with a closely related amino acid. Similarly, human patients with GABA transaminase deficiency disease also exhibit a small yet significant increase in β-alanine, along with a large increase in GABA (Medina-Kauwe et al., 1998). GABA levels were restored to normal in *pop2-1*/*pop2-1* mutants transformed with a wild-type gene. GABA transaminases typically transfer an amino group to pyruvate or α-ketoglutarate; in vitro assays confirm that recombinant POP2 transaminase removes an amino group from GABA and transfers it to pyruvate (Van Cauwenberghe et al., 2002).

Other intermediates in the GABA pathway were unaf-

ovules are attached. The tube carries two sperm at its tip, grows along the funiculus, and enters the micropyle opening of the ovule to reach the seven-celled embryo sac. Upon pollen tube reception, one sperm fertilizes the egg cell to yield a zygote, and the other fuses with the central cell to form the endosperm.
(C) *POP2* has 18 exons; *pop2-1* has a splice site acceptor mutation (exon 7, arrow); *pop2-2* and *pop2-3* have T-DNA insertions into introns 12 and 14, respectively.
(D) Phylogeny of class III transaminases (DNAStar, Madison, WI); Genbank accessions and amino acid substrates are indicated.
(E) Protein sequence of the conserved pyridoxal phosphate cofactor binding site (*). Gaps (dashes) were introduced for alignment, and residues identical (black) or similar (gray) in a majority of sequences are shaded.
(F) POP2 is a GABA transaminase in the GABA shunt (Shelp et al., 1999; Gibson et al., 1990).

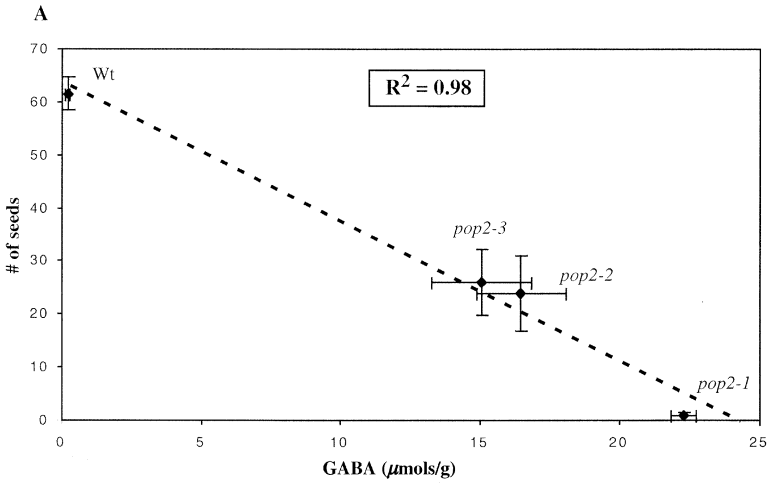


Figure 2. GABA in Floral and Leaf Tissues from Wild-Type and *pop2* Mutants

Values are the mean of triplicate determinations (±standard error), each with a sample size of five flowers, six fruits (seed yield), or 10 mg of leaf tissue. (A) Correlation between floral GABA and seed production. (B) GABA levels are less elevated in *pop2* leaves than in flowers.

B

	GABA		Glutamate	
	concentration (μmols/g)	Fold increase	concentration (μmols/g)	Fold increase
Wild type	0.09 ± 0.05	1	4.41 ± 1.28	1.0
pop2-1	2.09 ± 1.57	23	3.50 ± 0.74	0.79
pop2-2	1.39 ± 0.49	15	5.71 ± 0.17	1.3
pop2-3	0.99 ± 0.15	11	5.71 ± 0.76	1.3

fected by *pop2-1*. GABA transaminases convert GABA to succinic semialdehyde, which is rapidly oxidized to succinic acid or reduced to 4-hydroxybutyrate, completing the GABA shunt (Shelp et al., 1999; Figure 1F). Organic acid analysis of *pop2-1* flower extracts revealed normal succinate levels and only a 1.4-fold increase in 4-hydroxybutyrate (Table 1). Succinic semialdehyde was below detection limits, consistent with its highly reactive nature. Because it is produced as an intermediate in three other processes (tyrosine, butyrate, and vitamin B6 pathways), it is unlikely that *pop2-1* affects succinic semialdehyde availability. These assays strongly indicate that *POP2* encodes a GABA transaminase and that the *pop2-1* phenotype is a consequence of increased

Table 2. GABA Levels in Unpollinated Wild-Type and *pop2-1* Floral Organs

	POP2		*pop2-1*	
Organ	Nanomoles[a] (Concentration, μM[b])	Concentration Relative to the Stigma[c]	Nanomoles[a] (Concentration, μM[b])	Concentration Relative to the Stigma[c]
Stigma	0.07 ± 0.04 (20 ± 10)	1.0	3.7 ± 0.80 (1200 ± 300)	1.0
Style	0.21 ± 0.17 (60 ± 30)	3.0	5.1 ± 1.7 (1700 ± 500)	1.4
Septum	1.60 ± 0.24 (160 ± 20)	8.0	19 ± 3.0 (1900 ± 300)	1.6
Inner integument[d]	500 μM	25	24000 μM	20
Ovule/funiculus	0.16 ± 0.04 (30 ± 10)	1.5	8.9 ± 1.3 (1700 ± 300)	1.4
Ovary wall	1.1 ± 0.14 (110 ± 10)	5.5	21 ± 3.3 (2100 ± 300)	1.8
Whole pistil	0.68 ± 0.33 (20 ± 10)	1.0	51 ± 5.8 (1700 ± 200)	1.4
Pollen	5.4 ± 2.3 (270 ± 90)	14	22 ± 2.0 (1100 ± 200)	0.9

[a] Total GABA (nanomoles) in organs from 15 flowers; mean of three independent experiments (±standard error).
[b] Values incorporate measurements of wet organ fraction volumes.
[c] GABA concentration relative to that in stigmas of the same genotype.
[d] GABA in the inner integument cells, estimated by multiplying GABA concentration in the ovule/funiculus fraction by the relative fluorescence of the inner integument, ovule, and funiculus (inner integument signal accounted for 67% of the total in wild-type and 56% in *pop2-1*), and then adjusting to reflect the relative volume of the inner integument cells (~4%).

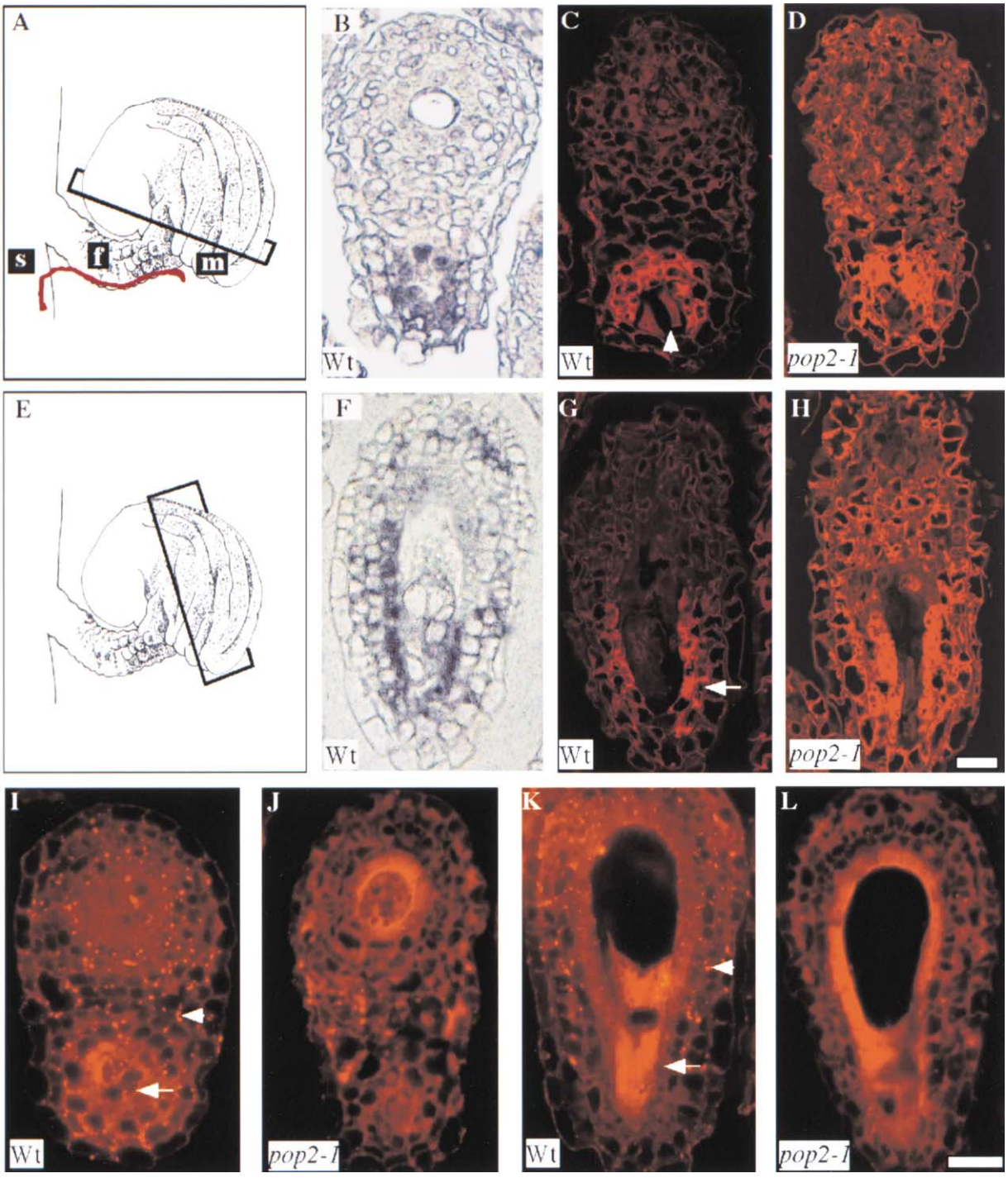


Figure 3. GABA and POP2 Immunolocalization

(A) Pollen tubes (red) emerge from the septum(s), navigate up the funiculus (f), and enter the micropyle (m). Sections of ovules from wild-type (B, C, F, G, I, and K) and *pop2-1* (D, H, J, and L); cross- and transverse sections were prepared as diagrammed in (A) and (E), respectively. Sections (B)–(D) and (F)–(H) were probed with anti-GABA and sections (I)–(L) with anti-POP2 antibodies. Detection of bound antibodies was with silver (B and F) or TRITC (C, D, G, H, and I–L). Large quantities of GABA were detected in a subset of inner integument cells (arrow) surrounding the micropyle (arrowhead; B, C, F, and G). Elevated GABA was apparent throughout *pop2-1* ovules (D and H). POP2 staining (I and J) shows typical mitochrondrial localization throughout the ovule (arrowhead), except for the subset of inner integument cells (arrow); mitochondrial staining was absent in *pop2-1* (I–L). Western blots (data not shown) of wild-type and POP2 extracts detected two bands: POP2 (present only in wild-type) and a crossreacting protein (in wild-type and *pop2*). The latter may be the source of the background cytoplasmic staining visible in *pop2* sections; no staining was observed when primary antibodies were omitted. Scale bars = 20 μm in (B)–(D) and (F)–(L).

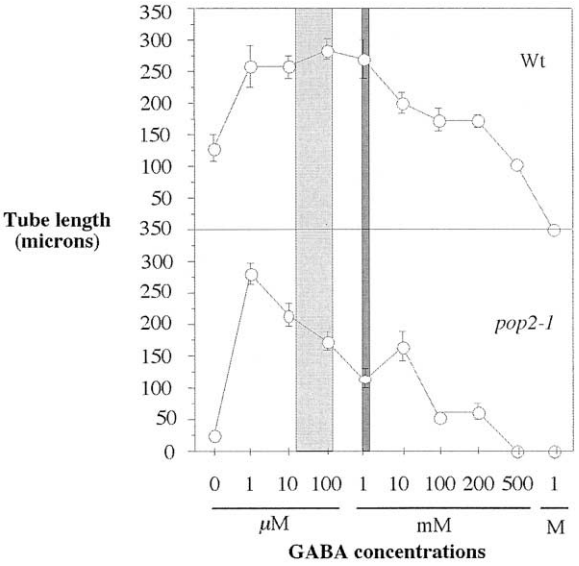


Figure 4. Length of In Vitro Grown Wild-Type (Wt) and *pop2-1* Pollen Tubes

The line connects mean lengths (circles); data were collected from ≥15 tubes at each GABA concentration. Light (wild-type) and dark gray (*pop2*) bars represent in vivo GABA concentrations of organs on the pollen tube path (stigma, style, and septum). Similar results were obtained in three independent experiments.

GABA, rather than alterations in the abundance of other GABA pathway compounds.

Developmental Consequences of GABA Accumulation

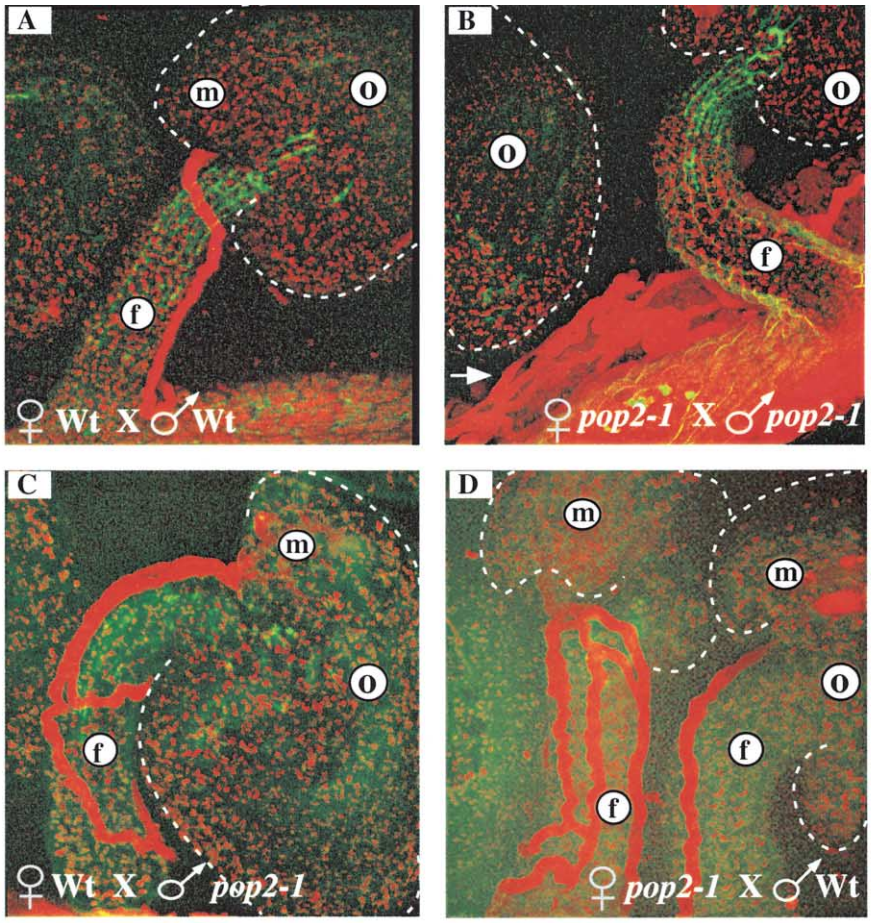
GABA accumulation was also apparent in *pop2-2* and *pop2-3*, generated by T-DNA insertions into *POP2* introns (Figure 1C), but was not observed in other infertile mutants, including *cer6-2* and *ms-1* (male-sterile) and *bel1* and *sin1* (female-sterile). Floral GABA levels and fertility were inversely correlated across *pop2* alleles (Figure 2A), indicating that pollen tube targeting is less efficient with increasing GABA. In addition, flowers on secondary *pop2-1* inflorescences accumulated less GABA than those on primary branches and exhibited a corresponding increase in fertility (7 μmol/g GABA and 18 seeds per flower versus 22 μmol/g GABA and 1 seed per flower). These results are reminiscent of the GABA concentration-dependent migration patterns of neuroblasts in vitro: proper migration was reported to occur at fM GABA concentrations; however, random motility was seen at μM levels (Barker et al., 1998).

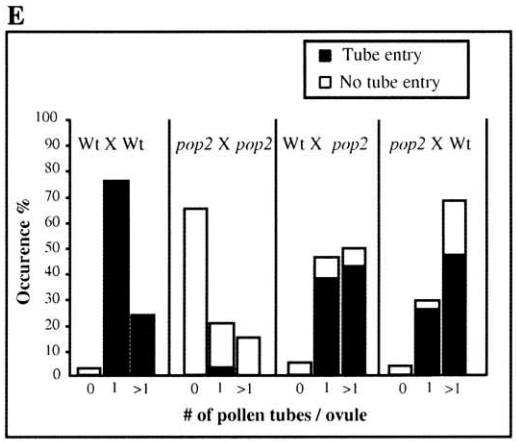
We found partially elevated GABA in *pop2* leaves (23-fold, 16-fold, and 11-fold in *pop2-1, -2,* and *-3*, respectively, Figure 2B). These increases were far less than those observed in flowers and are consistent with the lack of a vegetative phenotype in *pop2-1* mutants (Wilhelmi and Preuss, 1996). Exogenous GABA, however, can stimulate stem elongation at low levels and inhibit it at higher levels (Kathiresan et al., 1998). Transgenic tobacco plants with a 7-fold GABA increase in stems and a 2-fold increase in leaves have vegetative and floral defects, including short stems, narrow leaves, premature flower abscission, and interrupted pollen development (Baum et al., 1996). These phenotypes in tobacco resulted from the expression of a hypermorphic form of petunia glutamic acid decarboxylase (GAD) and might be due to a corresponding decrease in glutamate (18-fold in stems and 9-fold in leaves; Baum et al., 1996). *pop2*, in contrast, perturbed glutamate by less than 0.8-fold. These results, coupled with the highly specific effects on fertility, suggest POP2 is the principal GABA transaminase in flowers and that vegetative tissues may have a redundant enzyme. Furthermore, a second GABA transaminase that depends on α-ketoglutarate rather than pyruvate has been found in tobacco (Shelp et al., 1999).

GABA Concentrations Increase along the Pollen Tube Path

GABA is an extracellular signal for several cells; it is a neurotransmitter (Maitre et al., 2000) and a regulator of hormone secretion in endocrine organs (Gamel-Didelon et al., 2002; Satin and Kinard, 1998). Based on in vitro assays, it has also been proposed to function as a guidance cue for migrating neuroblasts (Barker et al., 1998). Plant cells can secrete GABA (Chung et al., 1992), and the *pop2* defect raises the possibility that GABA plays a signaling role in guiding pollen tubes. We examined the abundance of GABA throughout unpollinated pistils, separating tissues that represent different areas of the pollen tube path (Table 2, Figures 1A and 1B). Measurements of GABA content and cell volume showed that pollen tubes are exposed to low GABA at the stigma (20 μM), with an increasing amount of GABA in the style (60 μM), and even more GABA within the ovary locule, where the cells on the septum surface (160 μM) and ovary walls (110 μM) contain relatively high levels (Table 2).

On their way to the ovules, pollen tubes exit the septum, grow up the funiculus, and ultimately enter the micropyle that is flanked by integument cells (Figure 3A). We found 30 μM GABA in a fraction containing the entire ovule and funiculus, lower than that observed in the septum cells. Because further microdissection is challenging, we instead assayed the GABA distribution in fixed ovule sections by employing indirect immunofluorescence or histochemical staining (Figure 3). Serial sections showed that no other cell type stained as intensely as the inner integument (Figures 3B, 3C, 3F, and 3G; 305/305 sections from 25 pistils). This GABA focus was restricted to the subset of inner integument cells nearest the micropyle (Figures 3B, 3C, 3F, and 3G). The funiculus cells also contained substantial, albeit lower, GABA. Anti-glutamate antibodies yielded a more uniform distribution pattern, with staining throughout the ovule and funiculus (data not shown). With its function in GABA removal, POP2 levels are likely limiting in cells with high GABA. Indeed, anti-POP2 antibodies showed staining complementary to the GABA localization patterns (Figures 3I and 3K). These patterns suggest an important role for the subset of cells surrounding the micropyle. A role for these integument cells in *Paspallum orbiculare* pollen tube guidance was suggested previously (Chao, 1971). Based on relative cell volumes, we estimate that the inner integument cells contain 500 μM GABA, an enrichment of 25-fold relative to the stigma (Table 2). POP2 localization patterns were punctate,





F

♀	♂	# Offspring +/+	# Offspring pop2/+	# Offspring pop2/pop2	Probability
+/+	pop2/+	86	61	-	P > 0. 01
pop2/+	+/+	58	65	-	P > 0. 01
pop2/+	pop2/pop2	-	217	171	P > 0. 01
pop2/pop2	pop2/+	-	337	17	P < 0. 01

Figure 5. Pollen Tube Behavior near Wild-Type (Wt) and *pop2* Ovules

(A–D) Confocal images of pollen tubes (red) near wild-type (A and C) and *pop2-1* (B and D) ovules (outlined); crosses were on mature (A and B) or immature (C and D) pistils. (A) A wild-type tube successfully approaches a wild-type ovule. (B) Mutant tubes accumulate on the septum (arrow), and the ovule remains unfertilized. (C and D) Multiple pollen tubes approach ovules in immature pistils when either the female (C) or male (D) is *pop2*.

(E) Bar graph showing the number of times ovules were visited and fertilized by pollen tubes in reciprocal and self-crosses of immature pistils.

(F) Table showing offspring ratios from crosses of plants carrying *pop2-1* and *POP2* alleles. p, probability of obtaining by chance the observed variation from an expected 1:1 ratio; shading, significant deviation.

consistent with residence in the mitochondria (Figures 3I and 3K). Other GABA transaminases are also mitochondrial (Pinal and Tobin, 1998; Van Cauwenberghe et al., 2002), allowing GABA shunt products to readily enter the Krebs cycle.

In the mutant, the loss of POP2 protein causes a GABA

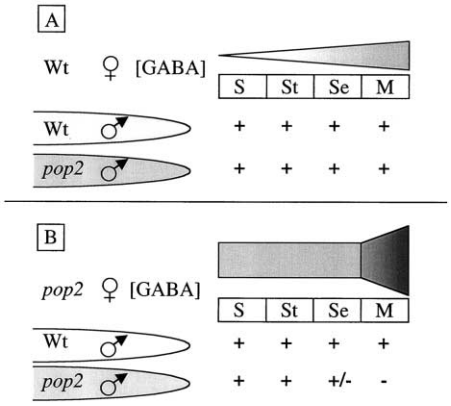


Figure 6. Model for GABA in Pollen Tube Growth and Guidance

Pollen tubes travel through several female tissues, including the stigma (S), style (St), septum (Se), and micropyle (M). (A) GABA (shading) is produced by wild-type female cells in a gradient that increases from stigma to micropyle (triangle). Wild-type and *pop2* pollen tubes sense this gradient by using POP2 to degrade GABA and exhibit normal growth and guidance (+). (B) In *pop2* mutant females, GABA levels are higher and the gradient between stigma and septum is abolished (filled rectangle); however, a gradient remains between the septum and the micropyle (trapezoid). POP2 activity enables wild-type pollen tubes to grow and navigate normally despite excessive GABA. *pop2* mutant pollen tubes are affected in two ways: (1) a portion exhibits growth arrest in the septum (+/−), and (2) the fraction that escapes arrest exhibits aberrant guidance to the micropyle (−).

enrichment of 60-fold in the stigma, 30-fold in the style, and 10-fold in the septum. These increases also alter the GABA gradient that gradually increases along the pollen tube path; *pop2* mutants instead lack the marked GABA difference between the stigma and septum and have an abrupt increase in GABA at the integument (Figure 3 and Table 2). In addition, *pop2* mutants have a GABA enrichment of 20-fold in ovary walls and 60-fold in the combined ovule and funiculus fraction (Table 2). Thus, the decrease in POP2 protein (Figures 3J and 3L) drives GABA throughout ovules to levels higher than those found in wild-type integument cells (Figures 4D and 4H; 153/153 sections from 25 pistils). Consequently, these cells may attract *pop2-1* pollen tubes to inappropriate locations, including the outer ovule surface. The *pop2-1* inner integuments cells still accumulate more GABA than their neighbors, suggesting a relatively higher capacity for GABA production.

Effect of GABA on Pollen Tube Elongation In Vitro

To provide direct evidence that GABA can affect pollen tube behavior, we incubated wild-type and *pop2-1* pollen on agarose medium containing GABA. For wild-type, up to 1–10 mM, GABA stimulated elongation, and higher concentrations were inhibitory (Figure 4). *pop2* tubes were hypersensitive, with growth stimulation peaking at 1–10 μM; higher levels, including those that stimulate wild-type, inhibited mutant tubes. Numerous mitochondria are located at pollen tube tips (Mascarenhas, 1993), and a reduction in POP2 transaminase activity in these organelles most likely accounts for the hypersensitivity of *pop2* tubes. Levels of GABA are elevated 4-fold in mutant pollen grains (Table 2) and are likely to increase in tubes after exposure to exogenous GABA. The complexity of the in vivo environment challenges direct comparisons with in vitro data; nonetheless, it is likely that the concentrations of GABA in the wild-type stigma, style, and septum stimulate pollen tube elongation, while the elevated GABA in *pop2* pistils is probably high enough to inhibit hypersensitive mutant tubes, but not their wild-type counterparts.

Providing a directional source of GABA in these assays did not alter growth polarity, nor was it altered in an *Arabidopsis* semi in vivo pollen tube guidance system (see Experimental Procedures), similar to that reported for other plants (Wu et al., 2000; Higashiyama et al., 2001). GABA alone, whether delivered from a filter disc or an agarose plug, was not sufficient to cause pollen tubes to turn toward the GABA source. Although this evidence suggests that free GABA is not sufficient to function as a directional cue, it is possible that these assays do not completely replicate in vivo conditions, particularly if responses require a series of developmental cues or tube attachment to a cellular substrate.

Pollen Tube Behavior near Wild-Type and Mutant Ovules

We monitored pollen germination, growth down the style and septum, elongation on the funiculus, entry into the micropyle, and fertilization in both wild-type and *pop2* pistils. Stage 12 buds (Bowman, 1993) were emasculated and allowed to mature for 24 hr before crossing to wild-type or *pop2*. After waiting 20–24 hr, several ovules at the stigma-end of multiple pistils were analyzed by confocal microscopy, and their association with stained pollen tubes was noted (47–128 ovules per cross). We found that pollen tubes efficiently targeted ovules if at least one parent was wild-type, consistent with previous measurements of seed yield (Wilhelmi and Preuss, 1996).

For self-crosses of wild-type, only 2% of the ovules remain unfertilized; of the remainder, 85% were visited by one pollen tube and 13% by more than one tube (Figure 5A; n = 128 ovules). Likewise, when wild-type pistils were crossed to *pop2* pollen or when *pop2* pistils were crossed to wild-type, we observed efficient growth to the ovules (n = 79 and 47 ovules, respectively), with no detectable defects in tube behavior. Thus, the high levels of GABA in a *pop2* pistil do not impair wild-type tube growth, potentially because these tubes can degrade excess GABA and thereby sense the gradient of GABA that leads from the septum to the micropyle. Similarly, an impaired ability to degrade GABA in mutant tubes does not affect targeting when normal GABA levels are encountered. In contrast, in self-crossed *pop2-1*, 76% of the ovules and funiculi were not associated with tubes (Figures 5B; n = 110 ovules). The growth of many *pop2* tubes was arrested on the septum surface, presumably due to GABA-mediated inhibition (Figure 5B). A subset of *pop2* tubes left the septum and approached an ovule but grew past the micropyle and accumulated on the funiculus or ovule exterior without fertilizing an egg. These data suggest a model in which high pistil GABA causes two defects when tubes are deficient in POP2 activity: (1) a premature arrest of tube elongation,

and (2) inappropriate guidance toward high sources of GABA emanating from the mutant septum, funiculus, and ovule exterior.

Pollen tube guidance is impaired when pistils are crossed several days before maturity (Kandasamy et al., 1994). We therefore sought subtler effects from *pop2-1* by crossing pistils just one day prior to maturation (stage 12 buds); like their mature counterparts, immature *pop2-1* pistils accumulated more GABA than wild-type (data not shown). Similar to mature pistils, self-crossed, immature wild-type ovules were fertilized efficiently with one tube (80%, Figure 5E; n = 77), and self-crossed, immature *pop2* pistils were infertile with few tubes near the ovules (65%, Figure 5E; n = 97). Intriguingly, reciprocal crosses with immature ovules revealed unusual behaviors—a large fraction were approached by multiple pollen tubes (Figures 5C and 5D). When the female parent was wild-type, 43% of the ovules were associated with multiple tubes (n = 85); when *pop2-1* was the female, a similar result was observed (48%, n = 90). This misdirection of multiple tubes to ovules directly reduced fertilization (Figure 5E). Interestingly, for the cross between wild-type pollen and immature *pop2-1* pistils, the pollen tubes showed a propensity to climb back down the funiculus or to stall on other tubes, a behavior that was much less common (8-fold and 10-fold, respectively) in the reciprocal cross.

These observations raise the possibility that the excess GABA that floods a *pop2* pistil can result in tube misdirection. In mature crosses, only *pop2* tubes are sensitive to high GABA. In contrast, crosses with immature pistils revealed defective behavior of both wild-type and mutant tubes. Although the molecular basis for the difference between mature and immature pistils remains unclear, it is likely that additional signals in mature pistils act to assure efficient pollen tube guidance. In their absence, the consequences of elevated GABA are more severe.

Genetic Consequences of the *pop2* Defect

Plant gametophytes have an active gene expression program; consequently, the genetically distinct gametophytes produced by a heterozygous *pop2* plant may behave differently. To further explore *POP2* functions, we performed reciprocal crosses between plants heterozygous for the *pop2* mutation and homozygous for the wild-type or mutant alleles; all of these crosses produced several seeds. We saw a highly significant deviation from the expected segregation ratio (30:1 observed, 1:1 expected) only when heterozygous *pop2*/+ pollen was used to pollinate a homozygous *pop2* pistil (Figure 5F). This abnormal segregation further supports the idea that, when exposed to excess GABA, wild-type pollen tubes are far better able to target the embryo sac than mutant tubes; it also strongly implies that POP2 protein is produced not only by diploid cells, but also during pollen tube growth. In contrast, when the pistil is heterozygous for the *pop2* defect, mutant tubes do not discriminate between wild-type and mutant eggs. This suggests that it is the abundance of GABA in the diploid tissue that surrounds the ovule, rather than the genotype of the haploid embryo sac itself, that is critical for successful guidance. Finally, control crosses involving wild-type and *pop2*/+ plants (Figure 5F) show that male and female gametophytes carrying *pop2* can function. Together, these results confirm that the GABA-mediated interactions that are perturbed in *pop2* involve the diploid pistil cells and the haploid pollen tubes.

Discussion

A Role for GABA in Pollen Tube Growth and Guidance

POP2 encodes a transaminase that degrades GABA. Our data suggest its activity modulates steady-state GABA levels in diploid female cells and the haploid pollen tubes, which function as a GABA source and sink, respectively (Figure 6). GABA rises to high levels in *pop2* flowers, causing a concentration-dependent defect in pollen tube growth, guidance, and fertility. The effects we report here implicate GABA as a direct or indirect (1) stimulator of pollen tube growth, (2) inhibitor, at high concentrations, of pollen tube elongation, and (3) signal that guides pollen tube navigation, most likely acting in concert with other guidance cues (Figure 6).

Evidence for direct stimulation and inhibition of tube growth comes both from in vivo observations and in vitro assays: at high concentrations GABA inhibits tube elongation and at low concentrations significantly stimulates it, a response reminiscent of GABA effects on stems (Kathiresan et al., 1998). *pop2* mutant tubes are hypersensitive to exogenous GABA, presumably because they lack the enzyme that degrades it. Consequently, although *pop2* tubes can successfully migrate along a wild-type septum where GABA levels are low and permissive for growth, the elevated GABA in *pop2* pistils causes reduced elongation (Figures 4, 5, and 6). Conversely, for wild-type tubes, neither wild-type nor mutant pistils have GABA levels that are in an inhibitory range, presumably because *POP2* tubes efficiently turn over GABA (Figures 4 and 6). The increasing GABA gradient between stigma and septum may exist to accelerate pollen tube growth; such factors may promote rapid growth in vivo that can rarely be recapitulated in vitro (Wu et al., 2000).

In vivo analyses raise the possibility that GABA also plays a role in pollen tube guidance. There is a GABA gradient from the septum to the micropyle, with GABA accumulating to high levels in the inner integument cells. In *pop2* mutants, GABA accumulates throughout the ovule, and the fraction of *pop2* pollen tubes that extends beyond the septum exhibit random and misguided growth. In addition, reciprocal crosses with immature pistils show differential behavior when male or female tissues carry *pop2*, indicating it is the source of GABA more than its absolute abundance that determines pollen tube behavior. Because GABA alone is not sufficient to guide tube growth in vitro, we propose that it most likely acts with other developmental signals to promote pollen tube guidance (Figure 6). In this model, pistil cells with low POP2 levels secrete a source of GABA that in combination with other factors attracts pollen tubes to specific targets. Wild-type pollen tubes sharpen this gradient by degrading intracellular GABA through the action of POP2. This activity is sufficient, even in a *pop2* pistil, to enable tubes to distinguish the micropyle from

the rest of the ovule. The process may be analogous to chemotaxis in aggregating *Dictyostelium* cells, where the degradation of cAMP allows them to maintain sensitivity toward the high cAMP levels in the aggregating center (Firtel and Meili, 2000). Conversely, the levels of GABA produced by wild-type integument cells may be sufficiently low, yet focused, for proper guidance of *pop2* tubes, even though these tubes are impaired in their ability to degrade GABA. The high GABA produced throughout *pop2* ovules, however, overwhelms mutant tubes, causing aberrant growth and guidance. GABA-based guidance signals may trigger changes in intracellular calcium, an ion known to regulate pollen tube targeting; similarly, the process of axon growth is positively and negatively regulated in response to a threshold of exogenous calcium (Kater and Miller, 1991).

The best-characterized function of GABA is the role it plays as an inhibitory neurotransmitter in the central nervous system. GABA mediates hyperpolarization of postsynaptic membranes by binding to $GABA_A$ and $GABA_C$ receptors (GABA-gated chloride channels); it also inhibits neurotransmitter release by interacting with $GABA_B$ receptors (G protein-coupled receptors; Pinal and Tobin, 1998). In the pancreas, GABA produced by β cells binds to $GABA_A$ receptors, stimulating insulin secretion from β cells (Brice et al., 2002) and glucagon secretion from α cells (Satin and Kinard, 1998). GABA is localized along the paths of migrating neurons and in their target destinations, and in vitro, GABA elicits concentration-dependent migratory responses from embryonic spinal and cortical neurons via GABA receptors and Ca^{2+} signaling (Behar et al., 2001). In addition, the growth of cerebral granule cells and cortical and tectal neurons is stimulated by GABA, an effect mediated by receptors in the plasma membrane (Hansen et al., 1987). In lower organisms, GABA stimulates growth as a nitrogen and/or carbon source (Prell et al., 2002). These diverse roles highlight GABA's potential as a signal for the growth and guidance of pollen tubes—motile cells that grow through several cellular environments to a defined target.

Regulating the Number of Pollen Tubes that Approach Each Ovule

Higher plants prevent more than one pollen tube from growing toward an ovule and thereby avoid polyspermy (Shimizu and Okada, 2000). This capability increases fitness by requiring pollen tubes to fertilize neighboring, genotypically different eggs and prevents the formation of polyploids with imbalanced paternal and maternal genomes. The limitation of one pollen tube per ovule may result from (1) terminating the release of attractive signals from the ovule once the embryo sac is fertilized (Higashiyama et al., 2001), or (2) initiating an inhibitory signal program, modulated by a pollen tube growing on the funiculus or by a fertilized embryo sac (Shimizu and Okada, 2000).

The crosses we performed with wild-type and *pop2* mutants reveal interesting insights into mechanisms that regulate the growth of multiple tubes on individual ovules (Figure 5E). (1) The ability of ovules to attract a single tube becomes increasingly established with development; reciprocal crosses between *pop2* and wild-type showed marked decreases in the number of multiple tubes per ovule as the pistil matured, suggesting an active system that blocks supernumerary tubes. (2) *pop2* is severely compromised in its ability to prevent multiple tubes from approaching an ovule, even when pistils are fully mature. (3) Male and female cells interact to prevent supernumerary pollen tubes; both *pop2* immature reciprocal crosses exhibited multiple tubes on the ovules. (4) In *pop2* mutants, tubes routinely grew in contact with each other over considerable distances, suggesting that increases in GABA cause a loss in repulsion or even an attraction between tubes. In contrast, analysis of the *maa* mutants, which also show multiple tubes per ovule, previously suggested that pollen tubes repel each other, with multiple tubes growing on opposite sides of the funiculus (Shimizu and Okada, 2000). These results together suggest that GABA either has a central role in inhibiting the growth of multiple tubes on an ovule or that elevated levels of GABA mask other signals essential for the prevention of multiple pollen tubes targeting a single ovule.

Small Molecule Signals for Plant Development

Free or conjugated amino acids often play important signaling roles in development. In abalone, L-tryptophan functions as a sperm attractant (Riffell et al., 2002), and in plants, amino acid conjugation of the tryptophan-related hormone indole acetic acid (IAA) is necessary for its storage, transportation, and regulation (Lasswell et al., 2000). Other amino acids defend against predation and disease. For example, L-canavanine, an arginine analog abundant in legumes, elicits antimetabolic effects when incorporated into insect proteins (Rosenthal, 1994). The neurologically active amino acid glutamate has been implicated in plant development. Agonists that target glutamate receptors cause defects in *Arabidopsis* development. Mutant plants resistant to these agonists and receptors that bind glutamate have been identified (Brenner et al., 2000; Lam et al., 1998). Similar to a highly conserved glutamate-based signaling mechanism, the prevalence of GABA signaling in plants and animals (Pinal and Tobin, 1998) suggests an ancient system that evolved prior to the divergence of the two kingdoms.

The later stages of pollen tube growth suggest a series of cell-signaling events. When pollen tubes travel along the septum, the tip is airborne, potentially allowing perception of volatile attractants. The distal tube regions are in contact with underlying female cells, allowing recognition of adhesive surface signals. Tubes navigate past fertilized ovules before reaching the funiculus of an unoccupied ovule, suggesting that fertilized ovules or pollen tubes emit inhibitory signals. Pollen tubes travel up the funiculus, usually adhering to the junctions between cell files, and at the top make an abrupt turn, disengage from cellular contacts, and grow through air space to the micropyle. These final stages of the pollen tube's journey require attraction, repulsion, and adhesion, analogous to the multiple cues required to guide axons to neural synapses (Palanivelu and Preuss, 2000).

GABA is likely one of several signals regulating pollen tube growth; others could derive from cell wall components, diffusible peptides, and products of plant metabolism. The behavioral distinction between *pop2* pollen

tubes in mature and immature pistils suggests these signals arise as pistils develop. Some repellent signals may reside in ovary walls; these tissues are adjacent to the septum and have fairly high levels of GABA yet, in wild-type, fail to attract tubes. While we demonstrated in vitro that GABA stimulates pollen tube growth, we did not recapitulate in vivo evidence for an attractive or repulsive role, suggesting that (1) the relevant signal may not be GABA itself, but a conjugated form of GABA or a molecule regulated by GABA, (2) the ability of pollen tubes to respond in vitro to exogenous GABA might first require interactions with other signals, and (3) GABA may need to be perceived by pollen from a highly focused source difficult to reproduce in vitro. GABA was implicated in pollen tube growth by directly assaying *pop2* extracts; consequently, it may prove worthwhile to use metabolic profiling of pollen and pistil fractions as a means to identify other small molecular signals.

Elucidation of GABA Signaling Pathways

Increases in glutamate decarboxlyase activity elevate GABA at the expense of glutamate pools in leaves and reproductive tissues (Baum et al., 1996). Although similar *Arabidopsis* mutations have not been characterized, it may be possible to alter GABA and glutamate pools by modifying one or more of the five glutamic acid decarboxylase (GAD) paralogs (Shelp et al., 1999). *POP2* encodes a pyruvate-dependent enzyme that is likely the predominant activity in flowers; an α-ketoglutarate-dependent transaminase most likely plays a role in leaves, limiting the vegetative effects of *pop2*. The *pop2* mutation is therefore somewhat unique, selectively targeting reproduction. Our results predict that an absence or reduction in pistil GABA levels would cause pollen tube growth and guidance defects; future work will test this model by searching for transgenic plants that contain low or no GABA.

A survey of the *Arabidopsis* genome did not uncover obvious receptors highly similar to those found in animals, yet the abundance of GABA and its response to stress signals strongly suggest that receptors are present. The absence of close animal homologs raises the possibility that plant GABA receptors have novel domains. Identifying these signaling components will provide a powerful tool for further dissecting the role of GABA in pollen tube growth and guidance, potentially revealing additional parallels between animal and plant GABA signaling.

Experimental Procedures

Plant Growth

Wild-type *Arabidopsis* ecotypes were *Landsberg erecta* (*Ler*), *Columbia* (*Col*), and *Wassilewskija* (*Ws*); *pop2-1* is *Ler*; *pop2-2* and *pop2-3* are *Ws*. *ms1* (CS75), *bel1* (CS3089), and *sin1* (CS3090) were from the *Arabidopsis* Biological Resource Center. Seeds were germinated on MS medium (Sigma) or soil, and plants were grown at 100 μE fluorescent light and 40% humidity.

Molecular and Genetic Analysis

DNA polymorphism segregation in sterile F_2 offspring from a wild-type (*Col*) $\times$ sterile (*Ler*) cross previously implicated *pop2* and *pop3* linked by 4.8 cM and 6 cM, respectively, to polymorphic markers (Wilhelmi and Preuss, 1996). Recombination breakpoint analysis defined a 25 kb *POP2* interval; RT-PCR identified GenBank number AF351125 as the *POP2* transcript. A *pop2-1* dCAPS marker (primers: F, GAGTTGTTTATGAATTTCCTGTATACCTAATGC and R, CCAATA ATGAGGGCAATCTGTGT; restriction digestion with EcoNI; Neff et al., 1998) confirmed complete linkage to sterility and strong linkage (4.5 cM) to an embryo lethal (*emb*) lesion. Unexpectedly, rare *pop2*/+ plants lacking the *emb* mutation yielded fertile:sterile offspring in a 3:1 ratio (1144:340, $p < 0.01$; χ^2 test), suggesting a single gene trait. The previously observed bias for *pop3* may have resulted from the restoration of fitness of plants carrying *emb* or other lesions. Additional evidence confirms *pop2-1* is sufficient for sterility: (1) analysis of molecular markers closely linked to *POP3* ($\leq$2 cM) identified sterile *pop2*/*pop2* plants that lacked *pop3* (7 plants /110 total), (2) backcrosses of *pop2*/*pop2*, *EMB*/*EMB* plants to wild-type yielded fertile:sterile F2 plants in a 3:1 ratio (1670:507, $p < 0.01$; χ^2 test), (3) other *pop2* alleles also cause sterility (Krysan et al., 1999; *pop2-2* and *pop2-3* were obtained from super pools 9, A, P64 and 1, B, P18, respectively), and (4) *POP2* transgenes restored *pop2-1* fertility: a 6 kb HindIII *POP2* genomic fragment was delivered with Agrobacterium GV3101 (pBI101 vector) to *pop2-1*/+ plants; three transformants homozygous mutant at the *pop2-1* locus were fully fertile, and three transformants heterozygous for *pop2-1* produced no sterile progeny.

Analysis of Floral Extracts

5–10 mg (amino acids) or 100 mg (GABA shunt components) of floral tissue was crushed in liquid nitrogen and extracted in methanol (62.5 μl), chloroform (150 μl), and water (37.5 μl); 10 μl of 10 mM n-butyric acid was added as a standard. The aqueous phase was dried with nitrogen gas and resuspended in 25 μl water (amino acids) or 375 μl water (GABA shunt components). Total free amino acids (Molecular Structure Facility, University of California, Davis) and 4-hydroxy butyrate and succinic semialdehyde (VU Medical Center, Netherlands; Gibson et al., 1990) were determined. GABA was not significantly different in *Ler* and *Ws* ecotypes.

Floral GABA was determined from pollen, collected as described for in vitro assays, and female tissues from 15 flowers were dissected with insect pins and a fine-tip forceps. Tissues were stored under methanol and pelleted to estimate their wet volumes, and amino acids were extracted as described above. Values represent the mean of three independent experiments; average volumes were 3 μl, stigmas; 3 μl, styles; 10 μl, septum; 10 μl, ovary walls; 5 μl, ovules and funiculi; 30 μl, whole pistils; and 20 μl, pollen.

Pollen Tube Growth Assays

Pollen from open flowers was suspended in modified growth medium (Li et al., 1999; 18% sucrose, 0.01% Boric acid, 2 mM $CaCl_2$, 1 mM $MgSo_4$). 2–3 μl of the pollen suspension was spotted on growth medium containing 0.5% purified agarose (Bio-Rad). Wild-type and *pop2* pollen were placed on the same petri dish and incubated at room temperature for $\sim$16 hr. Images were captured on a Zeiss Axiovert with Axiovision software. Pollen tube lengths were measured with NIH image (http://rsb.info.nih.gov/nih-image/index.html). For polarized growth assays, GABA concentrations between 10 nM and 10 μM were tested. For semi in vivo assays, pistils were pollinated and subsequently cut at the junction of the style and ovary chamber. Pollen tubes exited the excised stigma and style fragments, growing laterally on the surface of the supporting medium. GABA was delivered either from a filter disc or an agarose plug.

In Situ Localization

4 μm pistil sections, fixed with 2.5% gluteraldehyde, were prepared (Kandasamy et al., 1989) and probed with rabbit anti-GABA or rabbit anti-glutamate antibodies (1:100 dilutions, Sigma) or rabbit anti-POP2 antibodies, followed by goat anti-rabbit TRITC-conjugated (1:200 dilution, Sigma) or goat anti-rabbit horseradish peroxidase-conjugated (1:200 dilution, Amersham) secondary antibodies. Bound antibodies were observed with indirect immunofluorescence or the ECL chemiluminesence kit (Amersham). Images were captured on a Zeiss LSM-510 laser-scanning confocal microscope or Zeiss Axiovert (constant exposure, brightness and contrast settings). Wild-type and *pop2-1* pistils at equivalent developmental stages were sectioned and were processed on the same slide to eliminate experimental variation.

Pollen Tube Migration

Stage 12 flower buds (Bowman, 1993) were crossed either immediately (immature) or after 24 hr (mature). Pistils were harvested 20–24 hr later and the ovules, funiculi, and septum surface were viewed after removing one ovary wall. Pistils were mounted on glass slides and stained with filtered 0.1% Congo red (Grubler, Germany). Samples were viewed with a 63×, numerical aperture 1.2, water-corrected objective on a confocal microscope. For each pistil, five ovules were randomly selected for observation from the ten nearest to the stigma. Each tube in contact with the ovule and its funiculus was described, and ovules were categorized as unfertilized, or fertilized and (1) not associated with a pollen tube (0), (2) associated with one tube (1), and (3) associated with multiple tubes (>1).

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