The Plastidial Glyceraldehyde-3-Phosphate Dehydrogenase Is Critical for Viable Pollen Development in Arabidopsis[1][W]

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Plant metabolism is highly coordinated with development. However, an understanding of the whole picture of metabolism and its interactions with plant development is scarce. In this work, we show that the deficiency in the plastidial glycolytic glyceraldehyde-3-phosphate dehydrogenase (GAPCp) leads to male sterility in Arabidopsis (*Arabidopsis thaliana*). Pollen from homozygous *gapcp* double mutant plants (*gapcp1gapcp2*) displayed shrunken and collapsed forms and were unable to germinate when cultured in vitro. The pollen alterations observed in *gapcp1gapcp2* were attributed to a disorganized tapetum layer. Accordingly, the expression of several of the genes involved in tapetum development was down-regulated in *gapcp1gapcp2*. The fertility of *gapcp1gapcp2* was rescued by transforming this mutant with a construct carrying the *GAPCp1* cDNA under the control of its native promoter (*pGAPCp1*::*GAPCp1c*). However, the *GAPCp1* or *GAPCp2* cDNA under the control of the 35S promoter (*p35S*::*GAPCp*), which is poorly expressed in the tapetum, did not complement the mutant fertility. Mutant GAPCp isoforms deficient in the catalytic activity of the enzyme were unable to complement the sterile phenotype of *gapcp1gapcp2*, thus confirming that both the expression and catalytic activity of GAPCp in anthers are necessary for mature pollen development. A metabolomic study in flower buds indicated that the most important difference between the sterile (*gapcp1gapcp2*, *gapcp1gapcp2-p35S*::*GAPCp*) and the fertile (wild-type plants, *gapcp1gapcp2-pGAPCp1*::*GAPCp1c*) lines was the increase in the signaling molecule trehalose. This work corroborates the importance of plastidial glycolysis in plant metabolism and provides evidence for the crucial role of GAPCps in pollen development. It additionally brings new insights into the complex interactions between metabolism and development.

Glycolysis is a primary metabolic pathway whose main function is to oxidize hexoses to generate ATP, reducing power and pyruvate, and to produce precursors for anabolism (Plaxton, 1996). The major substrates fuelling glycolysis in plants are Suc and starch (Plaxton, 1996). Both metabolites are subjected to large diurnal changes in their concentrations that need to be coordinated with the rate of glycolysis to provide metabolic flexibility that can respond to differential demands of plant development and environmental stress acclimation. In plants, glycolysis occurs in both the cytosol and plastids that complicates our global understanding of this pathway and its interactions with development.

The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reversibly converts the glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate by coupling with the reduction of NAD^+ to NADH. In mammals, glycolytic GAPDH has been extensively characterized where, besides its pivotal role in energy production, it has been implicated in transcriptional regulation, DNA repair, signal transduction cascades, and apoptosis (Hara et al., 2005; Kim and Dang, 2005; Hara and Snyder, 2006; Colell et al., 2007; Min et al., 2007; Lee et al., 2009). These additional functions of glycolytic enzymes suggest that links between metabolic sensors and development could be established directly through enzymes that participate in metabolism (Kim and Dang, 2005). Nonglycolytic functions of plant GAPDHs have also been suggested either because of the nuclear localization of some isoforms or by the suppression of H_2O_2-mediated cell death in protoplasts (Baek et al., 2008; Holtgrefe et al., 2008). However, whether these postulated signaling functions of plant GAPDHs rely on their glycolytic activity remains unproven.

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A family of four genes encoding putative phosphorylating glycolytic GAPDHs (*GAPC1*, *GAPC2*, *GAPCp1*, and *GAPCp2*) is present in Arabidopsis (*Arabidopsis thaliana*; http://www.arabidopsis.org). GAPC1 and GAPC2 are cytosolic isoforms, while GAPCp1 and GAPCp2 are located in the plastids (Muñoz-Bertomeu et al., 2009). In spite of their low gene expression level compared to cytosolic GAPDHs, GAPCp down-regulation leads to drastic changes in the sugar and amino acid balance of the plant and causes arrested root development and sterility (Muñoz-Bertomeu et al., 2009). The arrested root development of *gapcp* mutants was attributed to a Ser deficiency, concluding that the major function of GAPCps in roots is to provide precursors for Ser biosynthesis (Muñoz-Bertomeu et al., 2009).

In this study, we investigated the role of GAPCps in pollen development. In flowering plants, pollen formation depends on differentiation and interaction of two cell types in the anther: the reproductive microsporocyte cells and the somatic cells that form the tapetum (Yang and Sundaresan, 2000). Here, we show that homozygous *gapcp* double mutants (*gapcp1-gapcp2*) are male sterile. Pollen obtained from *gapcp1-gapcp2* has collapsed shapes and is unviable. This is attributed to alterations in the tapetum layer as a result of the lack of GAPCp catalytic activity in anther tissues. The mechanism responsible for male sterility is studied, especially its relationships with sugar signaling. Our work corroborates the importance of plastidial glycolysis in metabolism and provides further evidence of the clear links between metabolism and development.

RESULTS

GAPCp Expression Is Increased in Reproductive Organs

The expression of the four Arabidopsis phosphorylating glycolytic GAPDH genes in roots, leaves, shoots, and flowers was investigated by quantitative reverse transcription-PCR (Q-RT-PCR; Table I). The expression of cytosolic *GAPC1* (At3g04120) and *GAPC2* (At1g13440) isoforms was lower in flowers than the calculated mean expression value of all organs studied. However, the expression of the plastidial *GAPCp1* (At1g79530) and *GAPCp2* (At1g16300) was higher in roots and flowers compared to the other organs. Consequently, the relative abundance of *GAPCp1* and *GAPCp2* in flowers was increased between 2 and 6 times compared to both *GAPC1* and *GAPC2*. Our data are in agreement with those found in the microarray databases where the relative expression of both *GAPCp1* and *GAPCp2* is increased by >4-fold in flowers compared to other glycolytic GAPDHs (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Specifically, *GAPCp1* expression is higher in early stages of flower development (stage 9) than in older stages (stage 15), especially in nongreen organs, such as petals, stamens, and carpels (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). Besides, *GAPCp1* is more highly expressed in the earlier stages of pollen development (uninucleate microspores and bicellular pollen) compared to mature pollen (Honys and Twell, 2004). Histochemical GUS analyses clearly corroborated that both *GAPCp1* and *GAPCp2* were expressed in stamens and carpels (Muñoz-Bertomeu et al., 2009; Fig. 1). In stamens, the GUS expression was specifically observed in the tapetum and at an early stage of pollen development (Fig. 1, A–F). An in silico analysis of the *GAPCp1* and *GAPCp2* promoters using the promomer tool (Winter et al., 2007) revealed that promoter regions of both genes were significantly enriched in consensus binding sequences present in important genes controlling anther development, such as the floral homeotic gene *AGAMOUS* (Supplemental Table S1). These data indicate that *GAPCp1* and *GAPCp2* promoters have relevant information required for anther gene expression.

T-DNA Insertions in *GAPCp1* and *GAPCp2* Trigger Male Sterility

Multiple independent T-DNA insertion mutant lines affecting *GAPCp1* and *GAPCp2* were obtained in a previous study (Muñoz-Bertomeu et al., 2009). Although none of the single mutants of either *GAPCp1* or *GAPCp2* presented a clear visual phenotype, different homozygous *gapcp* double mutants (*gapcp1gapcp2*) showed growth defects resulting in dwarf phenotypes and sterility (Muñoz-Bertomeu et al., 2009). The causes of this *gapcp1gapcp2* sterility were investigated. The stamens and pistils of *gapcp1gapcp2* look macroscopically normal when compared to wild-type plants (Fig. 2A). However, *gapcp1gapcp2* produced small siliques with no seeds (Fig. 2B). Crosses of wild-type pollen

Table I. *Expression analysis of glycolytic GAPDHs genes of Arabidopsis by Q-RT-PCR*

Values represent the relative expression (± SD) of each GAPDH in different organs compared to the mean expression value of all organs assayed. The ratio represents the relative abundance of plastidial GAPDHs in different organs compared to the cytosolic isoforms.

	Relative Expression				Ratio			
	GAPC1	*GAPC2*	*GAPCp1*	*GAPCp2*	*GAPCp1/GAPC1*	*GAPCp1/GAPC2*	*GAPCp2/GAPC1*	*GAPCp2/GAPC2*
Root	1.34 ± 0.28	1.37 ± 0.20	6.90 ± 0.50	4.38 ± 1.03	5.14	5.02	2.55	2.49
Leaf	0.93 ± 0.13	1.01 ± 0.00	0.12 ± 0.01	0.16 ± 0.04	0.13	0.12	0.27	0.25
Shoot	1.55 ± 0.10	0.71 ± 0.00	0.41 ± 0.05	0.95 ± 0.05	0.27	0.58	0.50	1.08
Flower	0.37 ± 0.00	0.74 ± 0.07	2.38 ± 0.06	1.67 ± 0.01	6.37	3.22	4.18	2.11

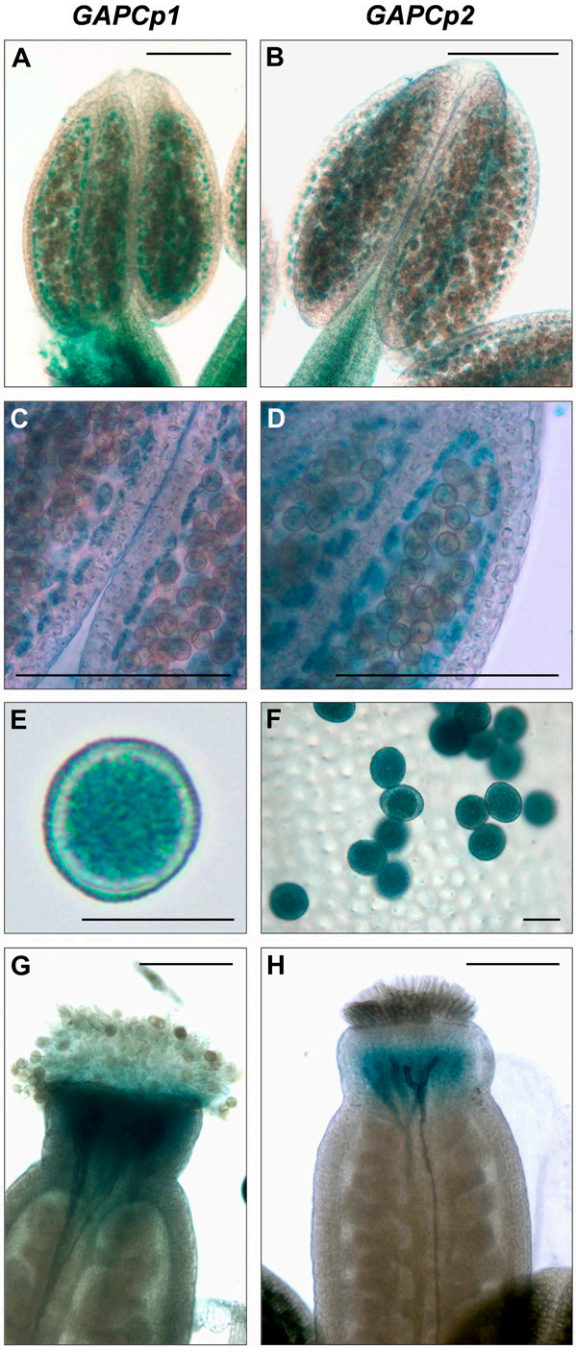

Figure 1. Expression analysis of *GAPCp1* and *GAPCp2* in flowers. Expression of *GUS* under the control of *GAPCp1* and *GAPCp2* promoters in stamens (A–D), pollen (E and F), and carpels (G and H). Bars = 0.15 mm (A–D), 20 μm (E and F), and 0.25 mm (G and H).

with *gapcp1gapcp2* ovules rescued the fertile phenotype, producing normal seeds and siliques (Fig. 2B). Yet, crosses of *gapcp1gapcp2* pollen with wild-type ovules produced small siliques with no seeds like those observed in the self-crossed *gapcp1gapcp2* (Fig. 2B). These findings indicate that *gapcp1gapcp2* mutations trigger male sterility. Scanning electron microscopy revealed that most of wild-type pollen had a uniform shape, but pollen from *gapcp1gapcp2* had shrunken and collapsed shapes (Fig. 3, A and B). In vitro germination assays demonstrated that pollen from three different homozygous *gapcp1gapcp2* alleles (*gapcp1.1/gapcp1.1 gapcp2.1/gapcp2.1*, *gapcp1.1/gapcp1.1*

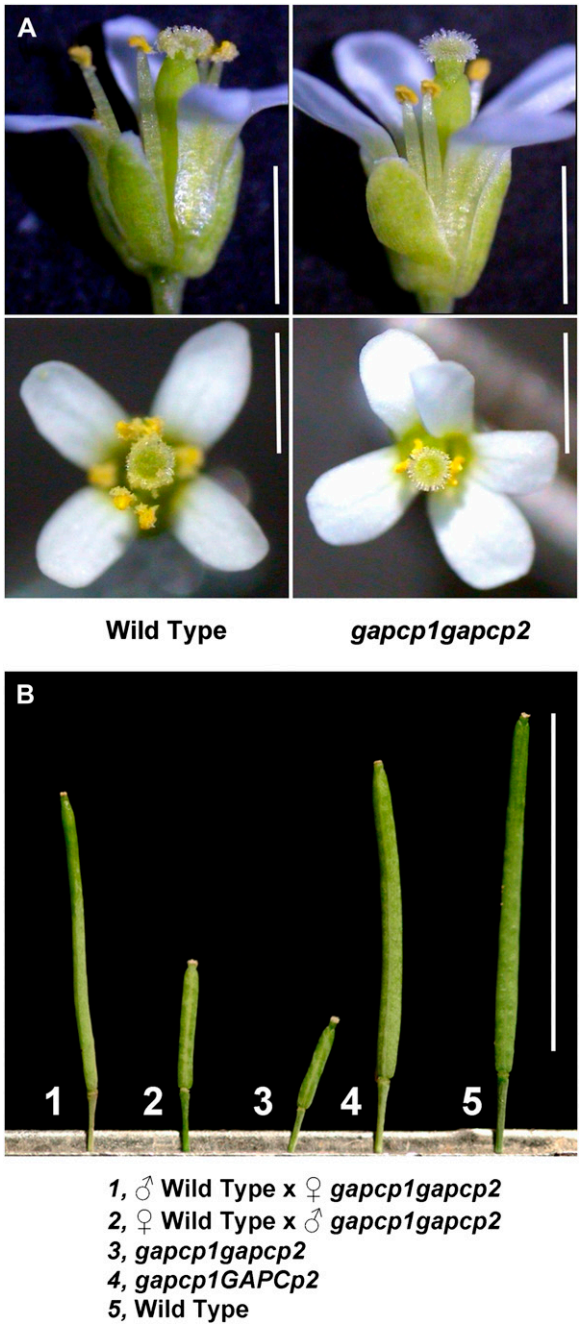

Figure 2. The *gapcp1gapcp2* double mutation causes male sterility. A, Side and top views of wild-type (left) and *gapcp1gapcp2* (right) flowers. Bars = 1.5 mm. B, Siliques obtained from (1) *gapcp1gapcp2* plants fertilized with wild-type pollen, (2) wild-type plants fertilized with *gapcp1gapcp2* pollen, (3) *gapcp1gapcp2* plants, (4) heterozygous *gapcp1GAPCp2* plants, and (5) wild-type plants. Bar = 1.5 cm.

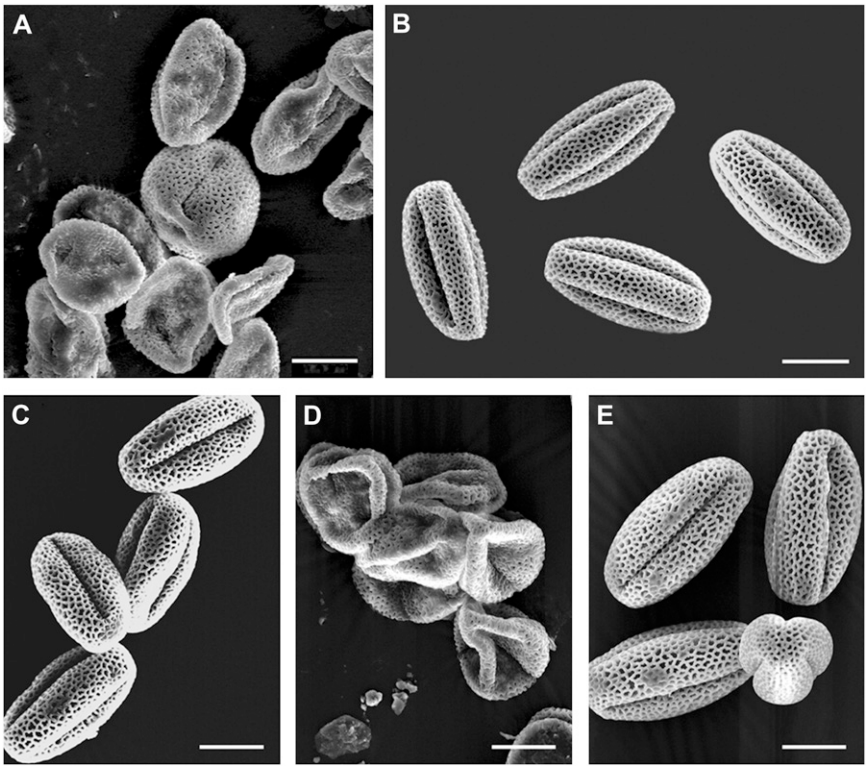

Figure 3. *gapcp1gapcp2* pollen shows aberrant phenotypes. Scanning electron micrographs of pollen isolated from *gapcp1gapcp2* (A), the wild type (B), *gapcp1gapcp2* transformed with a genomic GAPCp1 construct (C; *pGAPCp1*::*GAPCp1geno*), *gapcp1gapcp2* transformed with GAPCp1 cDNA under the control of the 35S promoter (D; *p35S*::*GAPCp1c*), and *gapcp1gapcp2* transformed with *GAPCp1* cDNA under the control of its native promoter (E; *pGAPCp1*::*GAPCp1c*). Bars = 10 μm.

gapcp2.2/gapcp2.2, and *gapcp1.1/gapcp1.1 gapcp2.3/gapcp2.3*) were unable to germinate (Table II), thus confirming that male sterility is genetically linked to both the *GAPCp1* and *GAPCp2* loci.

The transmission of the T-DNA insertion in self-fertilized *GAPCp1/gapcp1 gapcp2/gapcp2* or *gapcp1/gapcp1 GAPCp2/gapcp2* heterozygous plants was normal (1:2:1 wild type:heterozygous:double mutant Mendelian ratio; Muñoz-Bertomeu et al., 2009), indicating that *gapcp1gapcp2* pollen obtained from heterozygous plants germinate normally. In addition, the germination percentage of pollen from heterozygous plants (*GAPCp1/gapcp1.1 gapcp2.1/gapcp2.1 or gapcp1.1/gapcp1.1 GAPCp2/gapcp2.1*) did not differ from that of wild-type plants (Table II), confirming that male sterility in *gapcp1gapcp2* is caused by sporophytic effects. Thus, a defect in the anther development of the *gapcp1gapcp2* is probably the responsible for the pollen sterility observed in these plants.

Tapetum Development Is Impaired in *gapcp1gapcp2*

In order to explore the causes of pollen dysfunction in *gapcp1gapcp2*, we next investigated anther development. Sections of anthers and carpels of the wild type and *gapcp1gapcp2* were obtained from flowers at the same developmental stage (Fig. 4). At an early developmental stage (stages 5–6; Smyth et al., 1990), the tapetum layer in *gapcp1gapcp2* was completely disorganized (Fig. 4B). We also observed a delay in the ovule development of *gapcp1gapcp2* (Fig. 4D). At a later stage of flower development (stages 8–10), pollen grains were clearly observed in both the wild type and *gapcp1gapcp2*, although the malformations noted in the mutant pollen could already be distinguished (Fig. 4F). At this developmental stage, the ovules of *gapcp1gapcp2* developed normally, corroborating that this mutant has fertile ovules (Fig. 4H).

To further confirm that tapetum development was impaired in *gapcp1gapcp2*, the expression of a set of genes reported to be important in the tapetum and/or pollen wall development was studied. The following mRNA levels of both the wild type and *gapcp1gapcp2* were analyzed in closed buds (stages 5–10) by Q-RT-PCR: *MALE STERILITY1* (*MS1*; Wilson et al., 2001; Ito and Shinozaki, 2002), *DYSFUNCTIONAL TAPETUM1* (*DYT1*; Zhang et al., 2006), *ARABIDOPSIS THALIANA ANTHER7* (*ATA7*; Rubinelli et al., 1998), *LIPID TRANSFER PROTEIN12* (*LPT12*; Yang et al., 2007), and *GLYCINE RICH PROTEIN* At4g37900 (Yang et al., 2007). In agreement with the microscopy analyses, the expression of these genes was down-regulated in the *gapcp1gapcp2* inflorescences (Fig. 5). The expression of the glycolytic GAPDHs *GAPCp1*, *GAPCp2*, *GAPC1*, and *GAPC2* was also analyzed (Fig. 5). As expected, the expression of *GAPCp1* and *GAPCp2* was nearly undetectable in *gapcp1gapcp2*. Interestingly, the expression of the cytosolic *GAPC1* and *GAPC2* was up-regulated in the mutant, probably reflecting a compensatory effect for the lack of plastidial GAPDH activities.

Table II. *Germination rate of pollen from the wild type, heterozygous (g1.1/g1.1 G2/g2.1, G1/g1.1 g2.1/g2.1), double mutants (g1.1/g1.1 g2.1/g2.1, g1.1/g1.1 g2.2/g2.2, g1.1/g1.1 g2.3/g2.3), and transformed gapcp1 gapcp2 double mutant lines*

Mutant lines were transformed with the following constructs: *GAPCp1* cDNA fused to the 35S promoter (p*35S*::*G1c*), *GAPCp1* cDNA fused to the *GAPCp1* native promoter (*pG1*::*G1c*), *GAPCp1* genomic fragment fused to the *GAPCp1* native promoter (*pG1*::*G1geno*), and mutated *GAPCp1* cDNAs on the indicated amino acids (C236G, H263A, R318E, and K311A) fused to the *GAPCp1* native promoter. Values are the percentage of germination (± SD) compared to wild-type plants. The germination rate of wild-type plants is shown in parentheses. For simplicity, *g* stands for *gapcp* and *G* stands for *GAPCp*.

Genotype	Germination (%)
Wild type	100 (75%)
g1.1/g1.1 G2/g2.1	101 ± 12
G1/g1.1 g2.1/g2.1	95 ± 7
g1.1/g1.1 g2.1/g2.1	0
g1.1/g1.1 g2.2/g2.2	0
g1.1/g1.1 g2.3/g2.3	0
g1.1/g1.1 g2.1/g2.1 p35S::G1c	0
g1.1/g1.1 g2.1/g2.1 pG1::G1c	58 ± 8
g1.1/g1.1 g2.1/g2.1 pG1::G1geno	93 ± 36
g1.1/g1.1 g2.1/g2.1 pG1::G1c (C236G)	0
g1.1/g1.1 g2.1/g2.1 pG1::G1c (H263A)	0
g1.1/g1.1 g2.1/g2.1 pG1::G1c (R318E)	0
g1.1/g1.1 g2.1/g2.1 pG1::G1c (K311A)	97 ± 7

Expression and Catalytic Activity of GAPCp in Anthers Is Necessary for Mature Pollen Development

Since *GAPCp* mutations have pleiotropic effects on the plant, it might be argued that GAPCp deficiency could have an indirect effect on gametophyte development as a consequence of a general alteration of metabolism at the whole-plant level in *gapcp1gapcp2*. To answer this question, *gapcp1gapcp2* were transformed with *GAPCp* under the control of different promoters. The 35S promoter has a very low or no expression in the tapetum (Skirycz et al., 2007; Grienenberger et al., 2009). For this purpose, a *GAPCp1* or *GAPCp2* cDNA construct under the control of this 35S promoter (*p35S*::*GAPCp1c* or *p35S*::*GAPCp2c*) complemented all phenotypes except the male sterility in *gapcp1gapcp2* (Fig. 6, A and B). However, all the phenotypes of *gapcp1gapcp2*, including male sterility, could be complemented with a genomic construct (*pGAPCp1*::*GAPCp1geno*) of the *GAPCp1* locus (Fig. 6). When the *GAPCp1* cDNA was expressed under the control of its native promoter (*pGAPCp1*::*GAPCp1c*), full complementation of *gapcp1gapcp2* was once again achieved (Fig. 6).

Pollen morphology of *gapcp1gapcp2* transformed either with *pGAPCp1*::*GAPCp1geno* or *pGAPCp1*::*GAPCp1c* was similar to the wild type (Fig. 3, C and E). However, pollen from *gapcp1gapcp2-p35S*::*GAPCp1c* line presented the same morphological alterations as *gapcp1gapcp2* (Fig. 3D). The same was true for pollen viability. Only pollen from *gapcp1gapcp2* transformed either with *pGAPCp1*::*GAPCp1geno* or *pGAPCp1*::*GAPCp1c* was viable in an in vitro germination assay (Table II).

Several nonglycolytic functions for GAPDHs have been demonstrated in mammals and yeast (Hara and Snyder, 2006). To separate glycolytic functions from other additional putative functions of the GAPCps in anther development, amino acid substitutions were made to GAPCp1. GAPDHs are one of the most conserved proteins in living organisms. Sequence alignment of GAPCps with other GAPDHs from Arabidopsis identified several conserved amino acids already described in mammals as being essential for catalytic activity (Cys-236 and His-263), binding to the substrate glyceraldehyde-3-P (Arg-318) or putatively interacting with other regulatory proteins (Lys-311; Supplemental Fig. S1). Mutated versions of *GAPCp1* were used to transform *gapcp1gapcp2*. Fifteen (C236G),

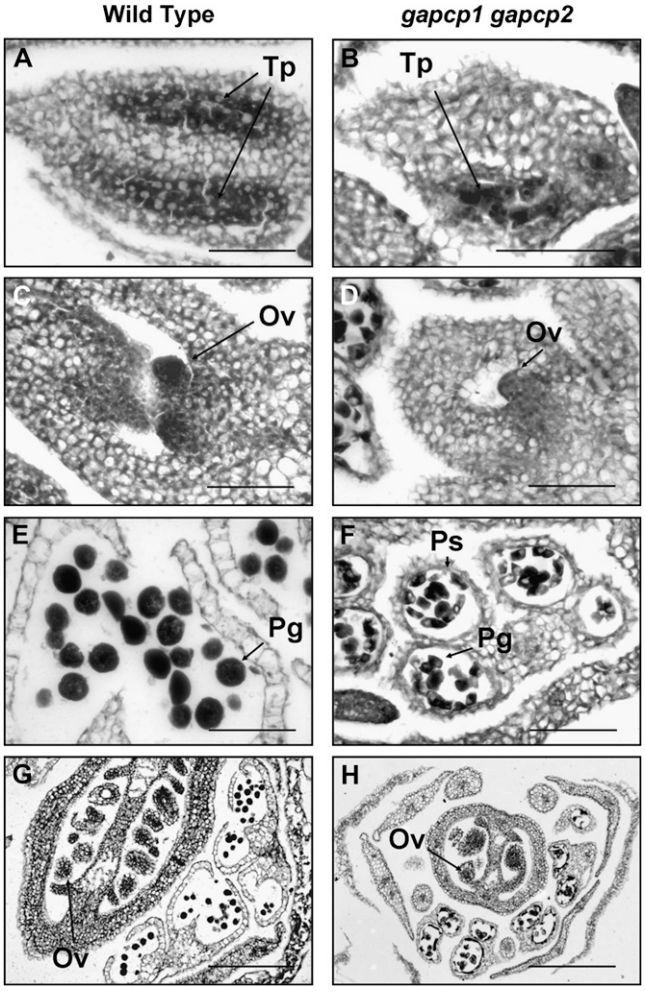

Figure 4. The tapetal cell layer is disorganized in *gapcp1gapcp2*. The micrographs show transverse sections of anthers and carpels at early (top) and late (bottom) developmental stages (stages 5 and 6 and stages 8–10). A and E, Wild-type anthers; B and F, *gapcp1gapcp2* anthers; C and G, wild-type carpels; D and H, *gapcp1gapcp2* carpels. Ov, Ovule; Pg, pollen grain; Ps, pollen sac; Tp, tapetum. Bars = 50 μm for A to D and F and 250 μm for G and H.

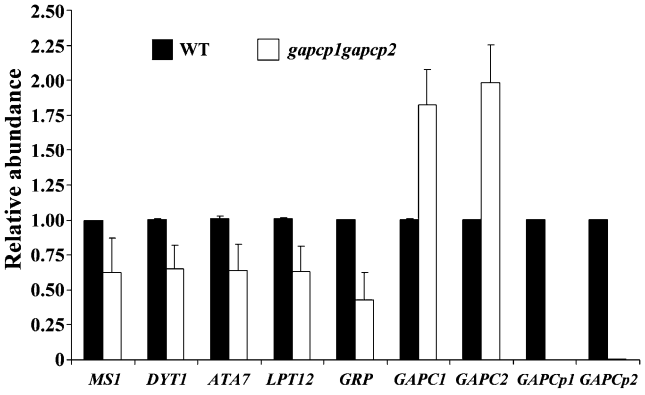

Figure 5. Expression of important genes in tapetum development is impaired in *gapcp1gapcp2*. Q-RT-PCR analysis of *MS1*, *DYT1*, *ATA7*, *LPT12*, *GLYCINE-RICH PROTEIN* (*GRP*), *GAPC1*, *GAPC2*, *GAPCp1*, and *GAPCp2* in wild-type (WT) and *gapcp1gapcp2* flower buds (stages 5–10).

10 (H263A), and 32 (R318E) independent T2 transgenic lines expressing the mutated isoforms that affect the glycolytic activity of GAPCp1 were studied. None of them was able to complement any of the phenotypes observed in the mutant (Fig. 7, A and B). Only the construct that carried a mutation that enables the catalytic function of GAPCp1 (K311A) was able to complement the male sterility phenotype and all the other phenotypes present in *gapcp1gapcp2* (Fig. 7, A and B). The in vitro germination assays confirmed that the K311A transformed lines were the only ones with viable pollen (Table II). Seedlings from a pool of three single insertion T3 homozygous lines were used to verify the expression of all the mutant constructs in Arabidopsis (Fig. 7C). This expression was compared to that of *GAPCp1* in the wild type and in *gapcp1gapcp2* complemented with *pGAPCp1*::*GAPCp1c*. The expression of all the mutated *GAPCp1* versions was similar but slightly lower than that found in the lines transformed with the wild-type *pGAPCp1*::*GAPCp1c*. No correlation between expression level of the mutated *GAPCp1* versions and complementation of the sterile phenotype was found.

Ser Supplementation Rescues All the Phenotypes Studied in *gapcp1gapcp2* But Not Male Sterility

gapcp1gapcp2 roots display a deficiency in Ser that causes root developmental arrest (Muñoz-Bertomeu et al., 2009). This was attributed to a limitation of 3-phosphoglycerate, the substrate for the phosphorylated pathway of Ser biosynthesis, in the mutant plastids. However, Ser levels in flower buds of *gapcp1gapcp2* were not reduced compared to control plants (Table III). There was even a clear tendency of an increase in the Ser content of the mutants. In accordance with these results, Ser supplementation rescued general growth but did not rescue the male sterility phenotype of *gapcp1gapcp2* (Fig. 8).

Metabolite Analysis in Flowers of *gapcp1gapcp2* and Transformed Lines

To investigate the effect of GAPCp deficiency on anther metabolism, flower buds of wild-type, *gapcp1gapcp2*, and *gapcp1gapcp2* transformed lines were harvested and the carbohydrate content measured. The starch and total soluble sugar contents in *gapcp1gapcp2* significantly increased by 48% and 18%, respectively, compared to the wild type (Fig. 9). Accordingly, the total sugars in *gapcp1gapcp2* buds significantly increased by 35% compared to the wild type. The carbohydrate contents also increased in buds of *gapcp1gapcp2* transformed with the *p35S::GAPCp1c* construct (sterile line), but they did not increase in those mutants transformed with the genomic construct (*pGAPCp1*::*GAPCp1geno*) of the *GAPCp1* locus (fertile line).

To further investigate the metabolic alterations in *gapcp1gapcp2*, a detailed analysis of the metabolites in flower buds was performed by a gas chromatography-mass spectrometry method (Table III; Supplemental Table S2). Although the content of Suc was slightly increased (14%) in *gapcp1gapcp2*, some other less abundant sugars, such as cellobiose, myoinositol, raffinose,

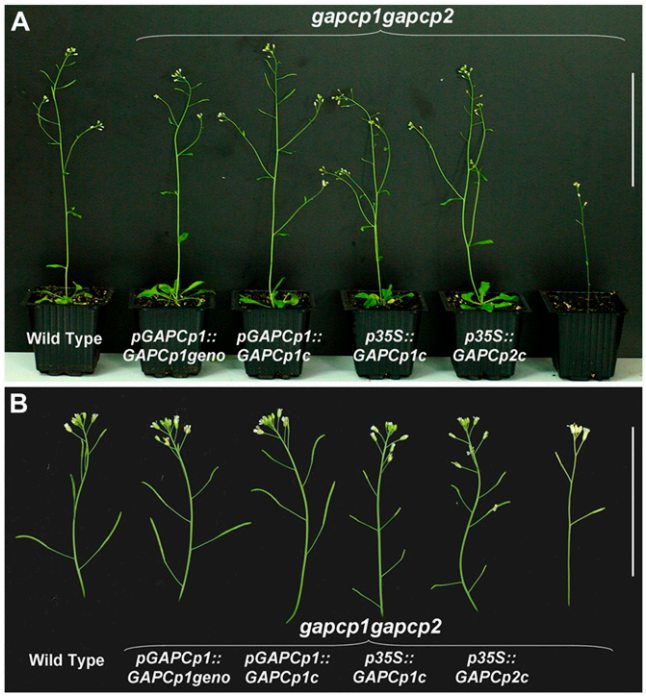

Figure 6. Phenotype of *gapcp1gapcp2* and transformed lines. A, Phenotype of wild-type plants, *gapcp1gapcp2* transformed with a genomic *GAPCp1* construct (*pGAPCp1*::*GAPCp1geno*), *gapcp1gapcp2* transformed with *GAPCp1* cDNA under the control of its native promoter (*pGAPCp1*::*GAPCp1c*), *gapcp1gapcp2* transformed with *GAPCp1* cDNA under the control of the 35S promoter (*p35S*::*GAPCp1c*), *gapcp1gapcp2* transformed with *GAPCp2* cDNA under the control of the 35S promoter (*p35S*::*GAPCp2c*), and *gapcp1gapcp2*. Bar = 10 cm. B, Close-ups to show differences in the siliques obtained from the different lines. Bar = 5 cm.

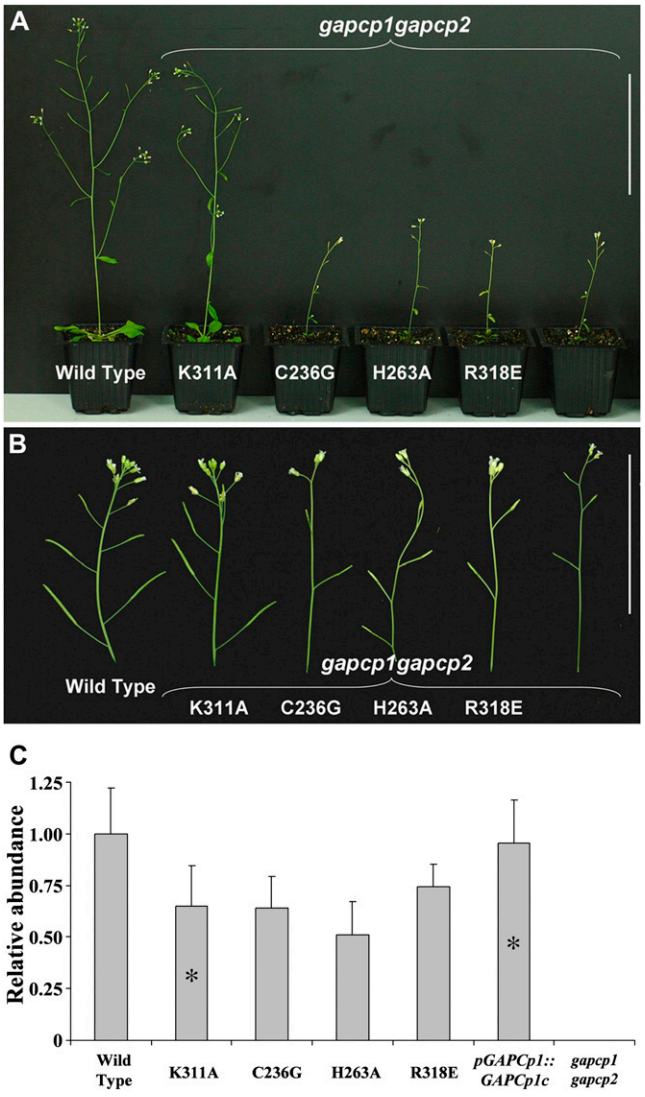

Figure 7. Mutated versions of *GAPCp1* do not complement *gapcp1-gapcp2* phenotypes. A, From left to right: the wild type, *gapcp1gapcp2* transformed with mutated versions of GAPCp1 (K311A, C236G, H263A, and R318E), and *gapcp1gapcp2*. Bar = 10 cm. B, Close-ups to show the differences in the siliques obtained from the different lines. Bar = 5 cm. C, Q-RT-PCR analysis of *GAPCp1* expression level in 3-week-old seedlings from the wild type, *gapcp1gapcp2* transformed with mutated versions or wild-type versions of GAPCp1 (K311A, C236G, H263A, R318E, and *pGAPCp1*::*GAPCp1c*), and *gapcp1-gapcp2*. Asterisk indicates line with fertile phenotype.

and trehalose, were increased by >2-fold compared to the wild type (Table III). The most important changes noted in *gapcp1gapcp2* were the increases in raffinose (6.5-fold increase) and in the signaling molecule trehalose (4.7-fold increase) compared to the wild type (Table III). The levels of the amino acids Ile and Trp also significantly increased by >2-fold in *gapcp1gapcp2* compared to the wild type.

The metabolite profiles of the sterile *gapcp1gapcp2-p35S*::*GAPCp1c* and *gapcp1gapcp2-p35S*::*GAPCp2c* lines were similar but not identical (Table III; Supplemental Table S2). However, both lines displayed significantly higher levels of cellobiose, myoinositol, raffinose, trehalose, and Trp than the wild type, as found in *gapcp1gapcp2* (Table III). In general, the metabolite changes measured in the *gapcp1gapcp2-p35S*::*GAPCp1c* line were weaker than those found in the *gapcp1-gapcp2-p35S*::*GAPCp2c* line, especially in the raffinose and trehalose levels (2.0- versus 4.7-fold increase in raffinose and 2.6- versus 3.9-fold increase in trehalose in *gapcp1gapcp2-p35S*::*GAPCp1c* and *gapcp1gapcp2-p35S*::*GAPCp2c*, respectively, compared to the wild type). The fertile *gapcp1gapcp2-pGAPCp1*::*GAPCp1geno* line did not fully phenocopy the metabolite profile of the wild type, although a clear trend of a similar profile to the wild type was observed (Table III; Supplemental Table S2). None of the metabolites measured varied >2-fold in the *gapcp1gapcp2-pGAPCp1*::*GAPCp1geno* line compared to the wild type. By comparing this fertile complemented line with the sterile lines (*gapcp1gapcp2*, *gapcp1gapcp2-p35S*::*GAPCp1c*, and *gapcp1gapcp2-p35S*::*GAPCp2c*), the most important change in the measured metabolites was the reduction of the trehalose content in the fertile line to levels resembling those observed in the wild type (only a 1.37-fold increase compared to 4.7-, 2.6-, and 3.9-fold increase in the sterile lines, respectively).

DISCUSSION

GAPCp Disruption Affects Tapetum Organization and Impairs Pollen Development

Mutations in the GAPCp genes cause male sterility. However, *gapcp1gapcp2* gametes obtained from heterozygous mothers are viable, indicating that the pollen sterility is not due to an alteration in the gamete itself, but to alterations in the cells participating in pollen development in *gapcp1gapcp2*. Several of the genes controlling tapetum development have been found to affect pollen viability and/or development (Colcombet et al., 2005; Mizuno et al., 2007; Yang et al., 2007; Zhu et al., 2008). The tapetum plays a major role in sporogenesis and is also critical in pollen wall and pollen coat formation (Paxson-Sowders et al., 2001; Dong et al., 2005; Yang et al., 2007). Pollen from *gapcp1gapcp2* presented severe alterations of the pollen wall that may suggest that the tapetum is not functioning properly in these mutants. The disorganization of the tapetum layer, along with the down-regulation of the genes involved in tapetum development in *gapcp1-gapcp2*, would support this idea.

GAPCp Activity Is Essential for Viable Pollen Development

GAPCp1 or *GAPCp2* cDNA under the control of the 35S promoter could complement all the *gapcp1gapcp2* phenotypes, except the male sterile phenotype. It is known that the 35S promoter has poor expression in

Table III. *Most important metabolite changes in flower buds of gapcp1gapcp2, gapcp1gapcp2 transformed with GAPCp1, or GAPCp2 cDNA under the control of the 35S promoter (p35S::GAPCp1c; p35S::GAPCp2c) and gapcp1gapcp2 transformed with a genomic GAPCp1 construct (pGAPCp1::GAPCp1geno) compared to the wild type*

Data are normalized to the mean response calculated for the wild type. Values are represented as the mean ± SE of seven to 10 independent determinations from three different transgenic lines. Values in parentheses are absolute values (μmol g fresh weight^{-1}). Asterisk indicates that the absolute value was not quantified. Those values that are significantly different to the wild type are set in bold, $P < 0.05$. For simplicity, *g* stands for *gapcp* and *G* stands for *GAPCp*.

	Wild Type	*g1g2*	*g1g2 p35S::G1c*	*g1g2 p35S::G2c*	*g1g2 pG1::G1geno*
Amino acids					
Ile	1.00 ± 0.12 (0.13)	**2.09 ± 0.35**	1.64 ± 0.28	**1.60 ± 0.23**	1.47 ± 0.20
Ser	1.00 ± 0.15 (2.49)	1.68 ± 0.30	**1.66 ± 0.22**	**1.58 ± 0.16**	1.29 ± 0.14
Trp	1.00 ± 0.15 (0.03)	**2.50 ± 0.41**	**2.03 ± 0.37**	**2.01 ± 0.11**	**1.95 ± 0.29**
Sugars and sugar alcohols					
Cellobiose	1.00 ± 0.09 (*)	**2.53 ± 0.48**	**1.69 ± 0.14**	**1.79 ± 0.25**	**1.66 ± 0.16**
Myoinositol	1.00 ± 0.04 (0.96)	**2.05 ± 0.25**	**1.48 ± 0.10**	**1.78 ± 0.11**	**1.21 ± 0.07**
Raffinose	1.00 ± 0.00 (0.01)	**6.50 ± 0.85**	**2.03 ± 0.04**	**4.72 ± 0.09**	**1.76 ± 0.03**
Suc	1.00 ± 0.04 (5.25)	**1.14 ± 0.05**	1.14 ± 0.10	1.04 ± 0.09	0.96 ± 0.04
Trehalose	1.00 ± 0.09 (0.16)	**4.68 ± 0.45**	**2.64 ± 0.26**	**3.92 ± 0.55**	**1.37 ± 0.05**

the tapetum layer of Arabidopsis (Skirycz et al., 2007; Grienenberger et al., 2009). As in our case, the Arabidopsis mutant in the *SOMATIC EMBRYOGENESIS RECEPTOR KINASE1* (*SERK1*) and *SERK2* genes, which are essential for tapetum development, could not be complemented when the *SERK* open reading frame was expressed under the control of the 35S promoter, but could be complemented with a genomic clone (Colcombet et al., 2005). These authors suggested that the noncoding sequences of the *SERK* locus might contribute to *SERK* function. By using the *GAPCp1* native promoter fused to the *GAPCp1* cDNA, we concluded that the *GAPCp1* cDNA expression is sufficient to complement the *gapcp1gapcp2* male sterility phenotype. Therefore, these results indicate that *GAPCp* expression in those cells participating in pollen development is necessary to achieve fertile pollen. They also suggest that the male sterility phenotype is not the result of a general alteration of the plant metabolism in *gapcp1gapcp2*, but the consequence of the lack of expression of GAPCp in anther specific tissues.

Recent works using mutant lines in other plastidic glycolytic enzymes downstream of GAPCp, such as pyruvate kinase (Andre and Benning, 2007; Baud et al., 2007) or enolase (Prabhakar et al., 2009), have not reported the drastic phenotype observed with *gapcp1-gapcp2*, especially the male sterility phenotype. We wondered if GAPCps could have additional nonglycolytic functions like those described for hexokinase (Rolland et al., 2006). Because of this, we transformed *gapcp1gapcp2* with GAPCps inactivated in their catalytic domain. Cys-236 has been described as being essential for catalytic activity (Didierjean et al., 2003). This Cys residue has also been reported to be important in signaling mechanisms such as *S*-nitrosylation-dependent apoptotic cell death cascades (Hara et al., 2005). To ensure that only the glycolytic functions of GAPCp were altered, other important amino acids for GAPCp1 glycolytic activity were also substituted (H263A and R318E; Didierjean et al., 2003). None of these mutant constructs was able to complement the male sterile phenotype of *gapcp1gapcp2*, thus confirming that the glycolytic activity of GAPCp is essential for correct pollen development. Therefore, GAPCps do not seem to have other functions other than catalytic activity in plants, or at least they are not involved in the phenotypes studied in *gapcp1gapcp2*.

Role of GAPCp in Flower Metabolism and Pollen Development

The metabolite profile of *gapcp1gapcp2* flower buds is clearly different from that of the wild type. These metabolite changes seem to be specific of GAPCp activity in flower organs since the *gapcp1gapcp2-p35S::GAPCp* lines, with a wild-type phenotype except for pollen viability, showed a similar metabolite profiling to *gapcp1gapcp2*, while that of the *gapcp1gapcp2-pGAPCp1::GAPCp1geno* line, with a fertile phenotype, was more similar to the wild type.

GAPCp disruption leads to alterations of pollen development that could be associated with changes in the metabolite profile of *gapcp1gapcp2* anthers. Since metabolite analysis was performed in flower buds, it could be argued that the changes observed do not represent those that may be occurring in the anther. If the only difference between the sterile *gapcp1gapcp2-p35S::GAPCp* and the fertile *gapcp1gapcp2-pGAPCp1::GAPCp1geno* lines is the differential expression of *GAPCp* in anthers, it could then be assumed that the metabolite differences between these two lines are a good indication of the changes occurring in this organ.

The main function of GAPCps in roots is to provide precursors for Ser biosynthesis (Muñoz-Bertomeu et al., 2009). Although Ser deficiency in some of the cellular types of anthers cannot be ruled out, the high levels of Ser in flowers of *gapcp1gapcp2*, as well as the noncomplementation of the male sterility by Ser supplementation, make this possibility unlikely. Thus,

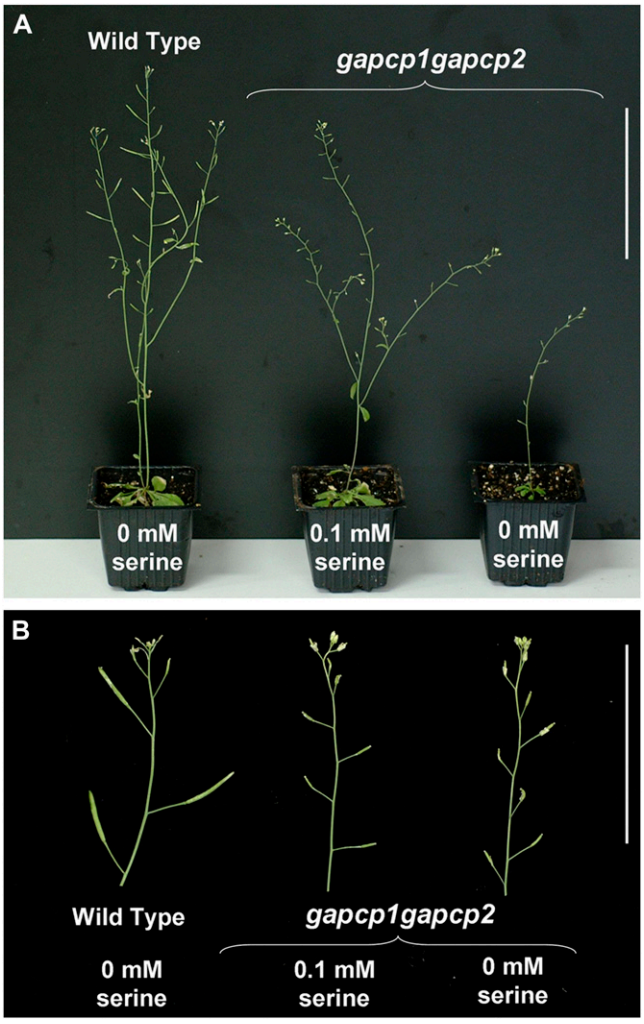

Figure 8. Ser supplementation does not rescue the male sterility phenotype of *gapcp1gapcp2*. A, *gapcp1gapcp2* seeds were germinated in plates supplemented with Ser, which were then transplanted to pots in a greenhouse. Plants were either irrigated or sprayed with 0.1 mM Ser. From left to right: the wild type and *gapcp1gapcp2* supplemented with Ser and *gapcp1gapcp2*. Bar =10 cm. B, Close-ups of the plants to show the differences in the siliques obtained from the different lines. Bar = 5 cm.

GAPCp main function in anthers seems to be different from that found in roots.

Carbohydrates not only play a critical role in anther and pollen development as an energy source, but are also necessary for pollen wall biosynthesis (Clement and Audran, 1995; Goetz et al., 2001; Woo et al., 2008). Starch and Suc levels increased in *gapcp1gapcp2* compared to controls. The impaired carbohydrate homeostasis in *gapcp1gapcp2* could affect pollen development. In this way, accumulation of starch in the pollen grain may well lead to reduced male fertility in tomato (*Solanum lycopersicum*; Nashilevitz et al., 2009). It has also been shown that the timing of callose wall formation and degradation is crucial for the normal pollen development (Izhar and Frankel, 1971; Warmke and Overman, 1972; Toller et al., 2008). The metabolism of callose could be affected in the *gapcp1gapcp2* tapetum as a consequence of the general alteration of the carbohydrate metabolism. In this case, the effect of GAPCp would be indirect.

The trehalose content in *gapcp1gapcp2* flowers increased by almost 5-fold compared to the control. The levels of this metabolite remained high in the sterile lines (*gapcp1gapcp2*, *gapcp1gapcp2-p35S*::*GAPCp1c*, and *gapcp1gapcp2-p35S*::*GAPCp2c*), but drastically dropped to approach wild-type levels in the fertile complemented line (*gapcp1gapcp2-pGAPCp1*::*GAPCp1geno*) where the starch and Suc levels were also similar to the wild type. The trehalose pathway is a central metabolic regulator of Suc and starch metabolism in plants (Wingler et al., 2000; Schluepmann et al., 2003; Paul, 2008; Paul et al., 2008; Schluepmann and Paul, 2009). Wingler et al. (2000) showed a strong activation of ADP-Glc pyrophosphorylase and accumulation of starch in response to trehalose feeding. Accordingly, the activities of enzymes involved in the synthesis of Suc and starch, such as ADP-Glc pyrophosphorylase, increased in *gapcp1gapcp2* (Muñoz-Bertomeu et al., 2009). Thus, the high levels of trehalose in *gapcp1-gapcp2* could be the direct consequence of GAPCp inactivity and might well lead to the observed changes in the main pools of carbohydrates. On the other hand, consensus is emerging in that trehalose also plays a central role in coordinating metabolism with development. Numerous effects on plant development have been observed as result of alterations of the trehalose

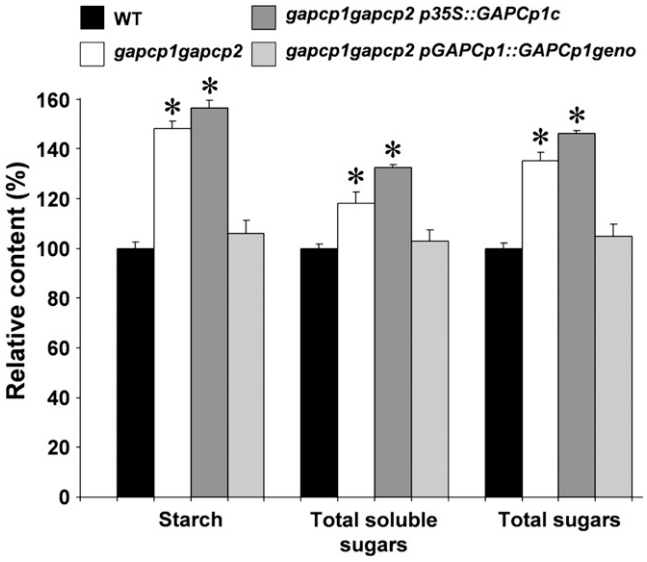

Figure 9. Starch and soluble sugar content in closed flower buds of *gapcp1gapcp2* and transformed lines. Carbohydrate content was measured in the wild type (WT), *gapcp1gapcp2*, *gapcp1gapcp2* transformed with *GAPCp1* cDNA under the control of the 35S promoter (*p35S*::*GAPCp1c*), and *gapcp1gapcp2* transformed with a genomic *GAPCp1* construct (*pGAPCp1*::*GAPCp1geno*). Values (relative content ± SD) were normalized to the mean value of the wild type in mg g fresh weight^{-1} (starch: 6.54 ± 0.16, soluble sugars 5.1 ± 0.1, total sugars 11.55 ± 0.25). Asterisk indicates significance at a P value <0.05.

pathway, which range from embryo and leaf development to inflorescence architecture (Eastmond et al., 2002; Pellny et al., 2004; Satoh-Nagasawa et al., 2006; Chary et al., 2008). Although trehalose has never been directly related to gametophyte development, overexpression of trehalose synthesis genes in maize (*Zea mays*) produced plants with sterility and seed formation-related problems (Almeida et al., 2007). In spite of the low expression of 35S promoter in anther tissues of Arabidopsis, expression of the trehalose-6-P synthase with the cauliflower mosaic virus *35S* promoter also produced mature plants with poor seed set (Schluepmann et al., 2003). Also, Arabidopsis plants treated with trehalose and Validamycin A, a strong inhibitor of the enzyme trehalase, accumulated high levels of trehalose and showed alterations in fruiting having much less siliques and no seeds (Muller et al., 2001). All these fertility problems could be attributed to male sterility, although in no instance was this possibility investigated by the authors.

The blockage in the plastidial glycolytic pathway in *gapcp1gapcp2* could be short-circuited through the cytosolic pathway, where the highly active cytosolic GAPDHs could metabolize the triose-phosphates. The increased expression of the cytosolic GAPDH isoforms in *gapcp1gapcp2* would support this idea. Then metabolites, such as pyruvate, 3-phosphoglycerate, or phospho*enol*pyruvate, could reenter the plastidial pathway through highly selective transporters present in the inner plastid membrane (Weber et al., 2005). The question that arises in the light of our data is why a deficiency in a minor GAPDH catalyzing a reversible reaction in plastids is able to provoke such drastic developmental alterations. Our results demonstrate a link between carbohydrate metabolism and pollen development. They also emphasize the importance of the GAPCps as regulators of the plant primary metabolism and as key enzymes in the connections between metabolism and development. Further studies will be needed to elucidate the role of specific metabolites, such as trehalose, in the network connecting GAPCp with pollen development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) seeds (ecotype Columbia-0) were supplied by the European Arabidopsis Stock Centre (Scholl et al., 2000) and by the Arabidopsis Biological Resource Center (http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/index.html). *gapcp1* and *gapcp2* single and double T-DNA mutant isolation and characterization were described by Muñoz-Bertomeu et al. (2009). Unless stated otherwise, seeds were grown under greenhouse conditions in pots filled with a (1:1, v/v) mixture of vermiculite and fertilized peat (KEKILA 50/50; Kekkilä Iberia) and were irrigated with demineralized water as needed. After a 4-d treatment at 4°C, seeds were placed in the greenhouse at 24°C for a 16-h day and at 17°C for an 8-h night photoperiod and were supplemented with artificial light as needed. Because *gapcp1gapcp2* are sterile, lines were maintained in heterozygosis (*gapcp1/GAPCp1 gapcp2/gapcp2* or *gapcp1/gapcp1 GAPCp2/gapcp2*). Homozygous *gapcp1gapcp2* plants were identified in segregating populations by the sterile phenotype. For Ser supplementation experiments, *gapcp1gapcp2* was grown in plates for 20 to 22 d and then transplanted to pots. Seeds were sterilized and sown on 0.8% agar plates containing one-fifth-strength Murashige and Skoog with Gamborg vitamins buffered with 0.9 g L^{-1} MES (adjusted to pH 5.7 with Tris). After a 4-d treatment at 4°C, seeds were placed in a growth chamber (SANYO; MLR-351H) at 22°C for a 16/8-h day/night photoperiod (100 μmol m^{-2} s^{-1}). Homozygous *gapcp1gapcp2* was identified in segregating populations by the short root phenotype after 8 to 10 d and was then subcultured on media containing 0.1 mM Ser for an additional 8 to 12 d. At day 20 to 22, plantlets were transplanted to pots as described above and were either irrigated or sprayed with 0.1 mM Ser.

Primers

All primers used in this work are listed in Supplemental Table S3.

Plasmid Constructs, Site-Directed Mutagenesis, and Plant Transformation

Standard methods were used to make the gene constructs (Sambrook and Russell, 2001). The cDNAs corresponding to genes *GAPCp1* (At1g79530) and *GAPCp2* (At1g16300) were placed under the control of the 35S promoter (*p35S*::*GAPCp1c* and *p35S*::*GAPCp2c*) by cloning them into a modified pGreen II plant transformation vector (Hellens et al., 1993) as described (Muñoz-Bertomeu et al., 2009). The construct *p35S*::*GAPCp1c* was used to exchange the 35S promoter by a 1.5-kb fragment corresponding to the native *GAPCp1* promoter (−1,521 to +18 relative to the *GAPCp1* translation start). The *GAPCp1* promoter was PCR amplified from BACs T8K14 (supplied by Arabidopsis Biological Resource Center) using PromAt1g79530FHindIII and PromAt1g79530RXhoI primers, introducing *Hin*dIII and *Xho*I sites and cloned in *p35S*::*GAPCp1c*, giving *pGAPCp1*::*GAPCp1c*. For the genomic complementation, a 5.5-kb fragment including 1,520 nucleotides upstream of the ATG of *GAPCp1* was obtained and cloned into a plant transformation plasmid derived from pFP101 named *pGAPCp1*::*GAPCp1geno* as described (Muñoz-Bertomeu et al., 2009). For gene promoter-reporter fusions, the promoter regions of gene *GAPCp1* and *GAPCp2* were fused to the *GUS* gene in pCAMBIA1303 as described (Muñoz-Bertomeu et al., 2009). Site-directed mutagenesis was performed with the Quikchange II XL site-directed mutagenesis kit (Stratagene) using the plasmid *pGAPCp1*::*GAPCp1c* as a template. The mutagenic primers used were the following: GAPCp1.C236G.F, GAPCp1.C236G.R, GAPCp1.H263A.F, GAPCp1.H263A.R, GAPCp1.K311A.F, GAPCp1.K311A.R, GAPCp1.R318E.F, and GAPCp1.R318E.R. All PCR-derived constructs were verified by DNA sequencing.

gapcp1gapcp2 was transformed with the different constructs using the floral dipping method (Clough and Bent, 1998) with *Agrobacterium tumefaciens* carrying pSOUP. Because *gapcp1gapcp2* was unable to produce seeds, we transformed the progeny of heterozygous plants (*gapcp1.1/gapcp1.1 GAPCp2/gapcp2.1* or *GAPCp1/gapcp1.1 gapcp2.1/gapcp2.1*) with the different constructs. Transformants were selected by antibiotic selection and *gapcp1gapcp2* verified by PCR genotyping using gene-specific primers (LP1 and RP1 for *GAPCp1* and LP3 and RP3 for *GAPCp2*) and left border primers of the T-DNA insertions (LB1_SAIL for *gapcp1.1* and LBa1 for *gapcp2.1*). Additional primers At1g79530FBProm and At1g79530R3Ex were used to differentiate genomic transgene insertion from the endogenous At1g79530. Single insertion homozygous T3 lines were selected for characterization.

Pollen Germination and GUS Activity Assays

In vitro pollen germination assays were done using the optimized solid medium described by Boavida and McCormick (2007). Experiments were performed with pollen from nine freshly opened flowers from three independent plants. In vitro pollen germination was examined under an Olympus BH-2 microscope. Images were collected from at least two different microscopy fields (about 200 pollen grains were scored) for each replicate. A pollen grain was classified as germinated if the pollen tube length was longer than the pollen grain diameter.

For GUS activity assays, plant organs were incubated in GUS buffer (100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 2 mM 5-bromo-4-chloro-3-indonyl-β-D-GlcUA [X-GlcA]; Duchefa) overnight at 37°C. The plant material was cleared either in 70% ethanol or Hoyer's solution before microscopy observation. At least six independent transgenic lines showed identical GUS-staining patterns and only differed in the expression level of

GUS. Images were acquired with a Leica DM1000 microscope and a Leica DC350 digital camera.

Microscopy

Floral buds of different developmental stages were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in phosphate buffer, pH 7, for 4 h at 4°C. After several washes with water, ethanol dehydration series were performed followed by washes in xylene. Afterward, samples were embedded in plasticized paraffin for 2 h at 60°C and subsequently the formed blocks were poured. Sections (8 μm maximum) were cut using a microtone (Microm), deparaffinized in xylene, and rehydrated and stained with Weigert's hematoxiline. A Leica DMLS microscope with a Leica DC300 digital camera was used for cytological observations.

For electron microscopy pollen was mounted on standard stubs and coated with gold-palladium prior to observation on a field emission microscope HITACHI S-4100.

Q-RT-PCR

Total RNA was extracted from roots, leaves, shoots, and closed buds using the RNeasy plant mini kit (Qiagen). RNA was treated with RNase free DNase (Promega). Then, 0.5 μg RNA was reverse transcribed using polyT primers and the first-strand cDNA synthesis kit for RT-PCR (Roche) according to the manufacturer's instructions. Real-time quantitative PCR reaction was performed using a 5700 sequence detector system (Applied Biosystems) with the Power SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. Each reaction was performed in triplicate with 1 μL of the first-strand cDNA in a total volume of 25 μL. The specificity of the PCR amplification was confirmed with a heat dissociation curve (from 60°C to 95°C). Efficiency of the PCR reaction was calculated and different internal standards were selected (Czechowski et al., 2005) depending of the efficiency of the primers. Relative mRNA abundance $\pm$ SD was calculated using the comparative Ct method according to Pfaffl (2001).

Metabolite Determination

Closed flower buds (stages 5–10) from plants grown in greenhouse conditions were used to determine metabolite content. Starch and total soluble sugars were determined with the ENZYTEC starch kit (ATOM). The levels of other metabolites were determined in derivatized methanol extracts by gas chromatography-mass spectrometry using the protocol defined by Lisec et al. (2006). The absolute concentrations of metabolites were determined by comparison with calibration standard curve response ratios of various concentrations of standard solutions as detailed by Roessner-Tunali et al. (2003).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At1g79530 (*GAPCp1*), At1g16300 (*GAPCp2*), At3g04120 (*GAPC1*), and At1g13440 (*GAPC2*).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Amino acid alignment of GAPCps with other members of the GAPDH family in Arabidopsis.

Supplemental Table S1. Enriched consensus sequences in the promoter regions of *GAPCp1* and *GAPCp2*.

Supplemental Table S2. Metabolite levels in flower buds of wild-type, mutant, and transformed lines.

Supplemental Table S3. Primers used in this work.

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