Plastidial Glycolytic Glyceraldehyde-3-Phosphate Dehydrogenase Is an Important Determinant in the Carbon and Nitrogen Metabolism of Heterotrophic Cells in Arabidopsis[1]

Armand D. Anoman, Jesús Muñoz-Bertomeu, Sara Rosa-Téllez, María Flores-Tornero, Ramón Serrano, Eduardo Bueso, Alisdair R. Fernie, Juan Segura, and Roc Ros*

Departament de Biologia Vegetal, Facultat de Farmácia (A.D.A., S.R.-T., M.F.-T., J.S., R.R.) and Estructura de Recerca Interdisciplinar en Biotecnologia i Biomedicina (A.D.A., S.R.-T., M.F.-T., J.S., R.R.), Universitat de València, 46100 Burjassot, Spain; Instituto de Biología Molecular y Celular de Plantas, Universidad Politécnica de Valencia-Consejo Superior de Investigaciones Científicas, 46022 Valencia, Spain (J.M.-B., R.S., E.B.); and Max Planck Institut für Molekulare Pflanzenphysiologie, 14476 Potsdam-Golm, Germany (A.R.F.)

ORCID IDs: 0000-0003-0043-2180 (A.D.A.); 0000-0002-2099-3754 (J.M.-B.); 0000-0002-6123-8173 (S.R.-T.); 0000-0002-9296-0070 (M.F.-T.); 0000-0002-0828-121X (E.B.); 0000-0001-7774-2676 (J.S.); 0000-0003-4254-8368 (R.R.).

This study functionally characterizes the Arabidopsis (*Arabidopsis thaliana*) plastidial glycolytic isoforms of glyceraldehyde-3-phosphate dehydrogenase (GAPCp) in photosynthetic and heterotrophic cells. We expressed the enzyme in *gapcp* double mutants (*gapcp1gapcp2*) under the control of photosynthetic (Rubisco small subunit *RBCS2B* [*RBCS*]) or heterotrophic (phosphate transporter *PHT1.2* [*PHT*]) cell-specific promoters. Expression of *GAPCp1* under the control of *RBCS* in *gapcp1gapcp2* had no significant effect on the metabolite profile or growth in the aerial part (AP). *GAPCp1* expression under the control of the *PHT* promoter clearly affected Arabidopsis development by increasing the number of lateral roots and having a major effect on AP growth and metabolite profile. Our results indicate that GAPCp1 is not functionally important in photosynthetic cells but plays a fundamental role in roots and in heterotrophic cells of the AP. Specifically, GAPCp activity may be required in root meristems and the root cap for normal primary root growth. Transcriptomic and metabolomic analyses indicate that the lack of GAPCp activity affects nitrogen and carbon metabolism as well as mineral nutrition and that glycerate and glutamine are the main metabolites responding to GAPCp activity. Thus, GAPCp could be an important metabolic connector of glycolysis with other pathways, such as the phosphorylated pathway of serine biosynthesis, the ammonium assimilation pathway, or the metabolism of γ-aminobutyrate, which in turn affect plant development.

Glycolysis is an essential primary metabolic pathway in most living organisms whose main function is to oxidize hexoses to provide ATP, reducing power and pyruvate, and to produce precursors for anabolism (Plaxton, 1996). In plants, glycolysis is particularly important because it is considered the predominant pathway that fuels the tricarboxylic acid cycle in mitochondria and is an important source of precursors for secondary metabolism, amino acids, and fatty acid biosynthesis (Plaxton, 1996). Plant glycolysis possesses some differences with respect to other organisms, which complicates its understanding (Plaxton, 1996). There are two glycolytic pathways operating in parallel in the cytosol and plastids, and both interact through highly selective transporters present in the inner plastid membrane (Weber et al., 2005), which may suggest that glycolytic intermediates are fully equilibrated in both compartments. However, the characterization of plastidial glycolytic mutants suggests that this may not always be the case, at least for some glycolytic intermediates in certain cellular types (Muñoz-Bertomeu et al., 2009, 2010). Furthermore, plants possess autotrophic photosynthetic cells and heterotrophic nonphotosynthetic cells, where the functions of the glycolytic pathway could be completely different. Besides, some of the glycolytic reactions in the chloroplast are identical to those in the Calvin-Benson cycle but operate in the opposite direction. For this reason, the functional significance of plastidial glycolysis has been questioned and still remains controversial,

especially in photosynthetic cells, where chloroplasts may lack one or several glycolytic enzymes (e.g. enolase and phosphoglycerate mutase; Van der Straeten et al., 1991; Andriotis et al., 2010; Prabhakar et al., 2010). For instance, plastidial glycolytic enolase has been demonstrated to have poor or no expression in chloroplasts, and mutant plants do not display drastic visible phenotypes (Prabhakar et al., 2010). Since the double mutant of enolase and the phospho*enol*pyruvate (PEP) transporter show a lethal phenotype and the PEP transporter single mutant phenotype is not lethal, it can be assumed that the plastidial enolase is functional in some cell types. However, Andriotis et al. (2010) concluded that, although plastids in developing Arabidopsis (*Arabidopsis thaliana*) embryos have an active lower part of the glycolytic pathway, the cytosolic glycolytic pathway suffices to support the flux from 3-phosphoglycerate (3-PGA) to PEP required for oil production. Other plastidial glycolytic enzymes of the upper and lower parts of the pathways have also been characterized. For instance, it has been demonstrated that pyruvate kinases, in the lower part of the glycolytic pathway, play an important role in seed oil biosynthesis (Andre and Benning, 2007; Andre et al., 2007; Baud et al., 2007), while triose-phosphate isomerase, in the middle part, is essential for the postembryonic transition from heterotrophic to autotrophic growth (Chen and Thelen, 2010). All these results highlight the specific functions of plastidial glycolytic enzymes in plants. However, our understanding of the general relevance of plastidial and cytosolic glycolysis in the plant is far from complete. Thus, it is a challenge to elucidate which enzyme isoforms are functionally important, in which cells there is a complete plastidial pathway, whether there is equilibrium of metabolic intermediates between the cytosol and the plastids, and in particular the exact role, regulation, and relative importance of different glycolytic isoforms in specific cell types and under different environmental conditions.

According to Arabidopsis genome databases (https://www.arabidopsis.org/), the number of annotated genes coding for different glycolytic enzyme isoforms varies greatly. Some enzymes are coded by few genes, such as triose-phosphate isomerase, which is represented by only two annotations in the databases, while others, such as phosphoglycerate mutase, is represented by more than 40 annotations. The Arabidopsis Information Resource database describes seven genes encoding phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GAPDH), four of which participate in glycolysis and three in the Calvin-Benson cycle. Two of the glycolytic isoforms (GAPC1 and GAPC2) are cytosol localized and two (GAPCp1 and GAPCp2) are localized in the plastid (Muñoz-Bertomeu et al., 2009). This may indicate the complexity of the GAPDH reaction in terms of the integration and regulation of enzyme activity. By following an antisense approach in potato (*Solanum tuberosum*), it was concluded that decreased GAPC activities exhibited no major changes in either whole-plant or tuber

morphology and that the enzyme plays only a minor role in the regulation of metabolism (Hajirezaei et al., 2006). By contrast, an Arabidopsis mutant in the GAPC1 isoform displayed delayed growth and several morphological alterations in siliques and seeds (Rius et al., 2008). The Arabidopsis double knockout *gapc* mutants, however, did not display any visible phenotype under normal growth conditions, but lack of enzyme activity decreased the oil content in developing seeds (Guo et al., 2012). Additional nonglycolytic functions for cytosolic GAPDHs have also been attributed in both plants and mammals (Kim and Dang, 2005; Zaffagnini et al., 2013). In Arabidopsis, GAPCs have been documented to be involved in the response to stress, for instance, participating in hydrogen peroxide signal transduction, in the response to cadmium toxicity, or in the immunity response (Guo et al., 2012; Vescovi et al., 2013; Han et al., 2015).

The plastidial GAPCps have been demonstrated to be critical for primary root growth and essential for microspore development (Muñoz-Bertomeu et al., 2009, 2010). It was hypothesized that the main function of GAPCps in roots is to supply 3-PGA to the phosphorylated pathway of serine biosynthesis (PPSB; Muñoz-Bertomeu et al., 2009). This hypothesis was later confirmed by the characterization of the PPSB (Cascales-Miñana et al., 2013). Double *GAPCp1* and *GAPCp2* mutants (*gapcp1gapcp2*) also display a drastic reduced growth of the aerial part (AP; Muñoz-Bertomeu et al., 2009), suggesting a role of the enzyme in this organ. Petersen et al. (2003) suggested that GAPCp is absent in the chloroplasts of angiosperms. However, Backhausen et al. (1998) postulated that GAPCp is essential for starch metabolism during the dark period in green and nongreen plastids. In this scenario, it would be involved along with phosphoglycerate kinase in the ATP production needed for starch metabolism (Backhausen et al., 1998).

In this work, we deepen the functional characterization of GAPCps in photosynthetic and heterotrophic organs by performing metabolomic and transcriptomic studies in *gapcp* mutants and in mutant lines in which the enzyme is specifically expressed in heterotrophic or in photosynthetic cells. We provide new insights concerning how GAPCp activity affects other metabolic pathways. We also identify genomic and metabolic targets responding to GAPCp activity in both roots and the AP. We conclude that GAPCp is an important link that connects metabolism with mineral nutrition and development in plants.

RESULTS

Specific Expression of *GAPCp* under the Control of the Rubisco Small Subunit Promoter Does Not Restore AP Growth But Complements *gapcp1gapcp2* Sterility

The phenotypic analysis of *gapcp1gapcp2* indicated that they display a drastic reduction not only of root growth but also of the AP when grown both on plates

and in greenhouse conditions (Supplemental Fig. S1; Muñoz-Bertomeu et al., 2009). This phenotype is observed in double homozygous mutants only. Single mutants (*gapcp1* or *gapcp2*) or mutant plants homozygous for one of the genes and heterozygous for the other are phenotypically indistinguishable from the wild-type plants (Supplemental Fig. S1), which demonstrates that *GAPCp1* and *GAPCp2* are redundant to one another. All the phenotypes of *gapcp1gapcp2* could also be complemented with constructs that carry the *GAPCp1* genomic sequence or the complementary DNA (cDNA) under the control of its native promoter (Muñoz-Bertomeu et al., 2009, 2010), which corroborates that *GAPCp1* can compensate the lack of *GAPCp2*. The metabolite profile of the *gapcp1gapcp2* AP indicated that 67% of analyzed metabolites were significantly altered and 45% were changed by more than 40% as compared with the wild type (Supplemental Table S1). These results point to the importance of GAPCp for metabolism in the AP. In this sense, mRNA for both GAPCp1 and GAPCp2 was clearly found in leaves, shoots, and reproductive organs (Muñoz-Bertomeu et al., 2009). Western blot using antibodies raised against an epitope of GAPCp isoforms further confirmed that the enzyme is present in the AP, although in lower amount than in roots (Supplemental Fig. S1).

In order to dissect the function of GAPCp in the AP, we expressed GAPCp1-GFP in *gapcp1gapcp2* under the control of the Rubisco small subunit *RBCS2B* promoter (*RBCS:GAPCp1*). According to the Bio-Analytic Resource for Plant Biology database (http://bar.utoronto.ca/), *RBCS2B* is mostly expressed in photosynthetic tissues in both dark and light conditions. It is also expressed in the shoot apex, shoots, seeds, and flowers but poorly expressed or nonexpressed in roots. GAPCp1 expression in the AP of different *gapcp1gapcp2* *RBCS:GAPCp1* lines was confirmed by quantitative real-time (qRT)-PCR, western blot using anti-GFP antibodies, and confocal microscopy, and those lines with the lowest expression in roots (*RBCS:G1.1*, *RBCS:G1.3*, and *RBCS:G1.4*) were further characterized (Fig. 1, A and B; Supplemental Fig. S2). At the adult stage, GAPCp1 expression in the AP was able to complement the sterile phenotype of *gapcp1gapcp2*, resulting in plants with siliques and fertile seeds (Fig. 1C). The developmental pattern of *gapcp1gapcp2* *RBCS:GAPCp1* was also altered as compared with *gapcp1gapcp2*, probably as a consequence of the fertile phenotype, displaying shorter shoots than *gapcp1gapcp2* (Fig. 1, C and D). A similar developmental pattern alteration was observed in the sterile *gapcp1gapcp2* *35S:GAPCp1* lines (Muñoz-Bertomeu et al., 2010) as compared with the wild type (Fig. 1, C and D). These results corroborate that GAPCp1 expression in reproductive organs is necessary for Arabidopsis fertility and confirm that GAPCp1 is active in *gapcp1gapcp2* *RBCS:GAPCp1* lines.

The presence of GAPCp1 in the photosynthetic cells did not recover the growth retardation of the rosette leaves and roots when plants were cultured in pots or on plates (Fig. 1, E–G). Similarly, the metabolite profile of the AP at the end of the light period in *gapcp1gapcp2* *RBCS:GAPCp1* lines overlapped that of *gapcp1gapcp2* according to principal component analysis (PCA; Fig. 2A; Supplemental Table S1). Other metabolites measured in *gapcp1gapcp2* *RBCS:GAPCp1* lines, such as starch, total soluble sugars, and proteins, were also not significantly different from *gapcp1gapcp2* (Fig. 1, H and I). In order to investigate whether GAPCp1 expression performed a function in photosynthetic cells under growth conditions where glycolysis is more important, for instance at night or when photosynthetic activity is inhibited, we analyzed the metabolite profile under dark conditions and in the presence of high concentrations of Suc, which inhibits photosynthesis (Fig. 2, B and C; Supplemental Table S2). Similar to samples obtained at the end of the light period, *gapcp1gapcp2* *RBCS:GAPCp1* lines displayed a metabolite profile that overlapped with *gapcp1gapcp2* in both darkness and under growth medium containing Suc (Fig. 2, B and C; Supplemental Table S2). Lack of metabolic and growth complementation in the rosette of *gapcp1gapcp2* *RBCS:GAPCp1* lines suggested that GAPCp1 expression in photosynthetic cells is not functionally important in spite of the drastic phenotypes observed in this organ in *gapcp1gapcp2*.

According to Tukey's posthoc test of the ANOVA of the first principal component, the metabolite profile of the AP of *gapcp1gapcp2* lines expressing GAPCp1-GFP under the control of *35S* (*35S:GAPCp1*) is similar to that of the wild type but differs from that of *gapcp1gapcp2* and *gapcp1gapcp2* *RBCS:GAPCp1* (Fig. 2A; Supplemental Table S1). In *gapcp1gapcp2* *35S:GAPCp1* lines, 14 of the 28 metabolites altered in *gapcp1gapcp2* recovered the wild-type levels and seven changed in the opposite direction to that in *gapcp1gapcp2*, which is suggestive of an overexpression effect. Since *35S:GAPCp1* is expressed in both photosynthetic and nonphotosynthetic cells (Fig. 1B), we postulated that lack of GAPCp activity in the latter cells could affect metabolism in the AP.

Expression of GAPCp under the Control of the Phosphate Transporter Promoter Modifies the AP and Root Metabolite Pattern and Partly Complements *gapcp1gapcp2* Phenotypes

According to the microarray databases and the literature, the phosphate transporter *PHT1.2* (*PHT*) promoter directs gene expression in root hair, epidermal, and cortex cells, with weak expression in rosette leaves (Mudge et al., 2002; Nussaume et al., 2011; Kirchsteiger et al., 2012; http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). For this reason, we next expressed GAPCp1-GFP under the control of the *PHT1.2* promoter (*PHT:GAPCp1*). Several lines with different *GAPCp1* mRNA expression levels in roots and the AP were obtained (Fig. 3A). Two of these lines (*PHT:G1.2* and *PHT:G1.5*) displaying high *GAPCp1* expression in
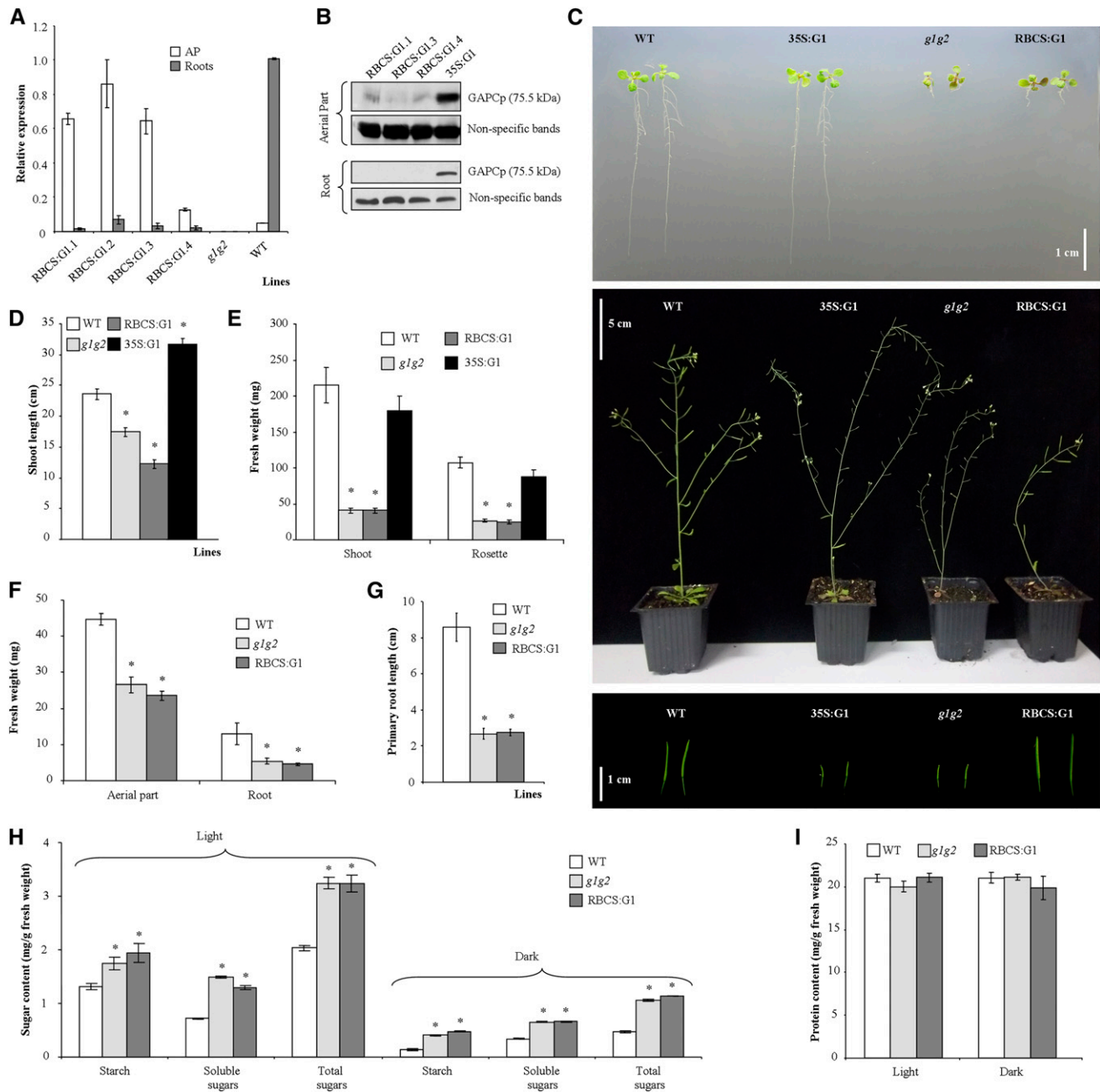
Figure 1. Molecular and phenotypical analysis of *gapcp1gapcp2* (*g1g2*) expressing GAPCp1-GFP under the control of the *RBCS* promoter (RBCS:G1). A, qRT-PCR analysis of *GAPCp1* in the APs and roots of several 18-d-old RBCS:G1 lines as compared with the wild type (WT) and *g1g2*. B, Immunoblot showing GAPCp1 expression in the APs and roots of three representative RBCS:G1 lines as compared with *g1g2* expressing GAPCp1 under the control of the *35S* promoter (35S:G1). Protein gel-blot analysis was performed using anti-GFP antibodies. Nonspecific bands are shown as a sample loading control at bottom. C, Morphology of seedlings (top), adult plants (middle), and siliques (bottom) from wild-type, 35S:G1, *g1g2*, and RBCS:G1 plants. D and E, Shoot length (D) and fresh weight of shoots and rosette leaves (E) of plants grown in the greenhouse. F to I, Fresh weight (F), root length (G), starch and total soluble sugar contents (H), and protein content (I) of wild-type, *g1g2*, and RBCS:G1 lines grown for 18 d on plates. In A, H, and I, values are means ± SE of three independent biological replications. In D to G, values are means ± SE of three independent transgenic lines ($n \geq 36$ plants). Asterisks indicate significant differences as compared with the wild type ($P < 0.05$).

roots and low expression in the AP were selected for further characterization. Western blots using anti-GFP antibodies confirmed that GAPCp1 was mainly

expressed in roots, although it could also be detected in the AP (Fig. 3B). In any case, the GAPCp content in the AP was much lower than in the selected *gapcp1gapcp2*
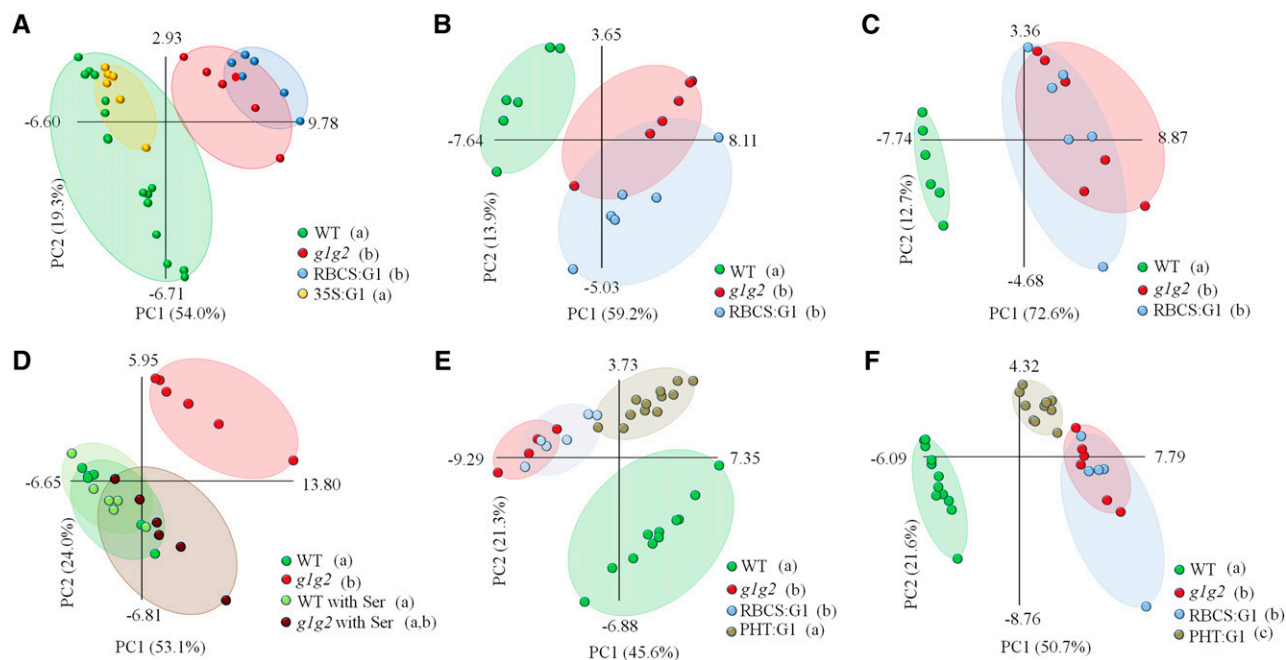
Figure 2. PCA of the metabolite profiles of different GAPCp mutant lines. Data from the gas chromatography-mass spectrometry analysis were evaluated using PCA, with the two first components accounting for at least 67% of total metabolic variance. Values in parentheses give the relative contribution of each component to the total variance observed in the data set. The first principal component was also used for the ANOVA of different lines, with Tukey's posthoc tests to differentiate between the lines. A, The AP at the end of the light period. According to Tukey's test, two different subsets are established: subset 1 (wild type [WT] and *gapcp1gapcp2* 35S:GAPCp1 [35S:G1] [a]) and subset 2 (*gapcp1gapcp2* [g1g2] and g1g2 RBCS:GAPCp1 [RBCS:G1] [b]). B, The AP at the end of the dark period. According to Tukey's test, two different subsets are established: subset 1 (wild type [a]) and subset 2 (*g1g2* and RBCS:G1 [b]). C, The AP at the end of the light period in medium supplemented with 2% (w/v) Suc. According to Tukey's test, two different subsets are established: subset 1 (wild type [a]) and subset 2 (*g1g2* and RBCS:G1 [b]). D, The AP at the end of the light period in medium supplemented with 0.1 mM Ser. According to Tukey's test, two different subsets are established: subset 1 (*g1g2* with Ser, wild type, and wild type with Ser [a]) and subset 2 (*g1g2* and g1g2 with Ser [b]). E, The AP at the middle of the light period. According to Tukey's test, two different subsets are established: subset 1 (wild type and *g1g2* PHT:GAPCp1 [PHT:G1] [a]) and subset 2 (*g1g2* and RBCS:G1 [b]). F, Roots at the middle of the light period. According to Tukey's test, three different subsets are established: subset 1 (wild type [a]), subset 2 (*g1g2* and RBCS:G1 [b]), and subset 3 (PHT:G1 [c]). Each ellipse includes all the data points of the same line being analyzed (cover hull).

RBCS:GAPCp1 lines (Fig. 3B). The *PHT:GAPCp1* construct complemented the delayed AP growth of *gapcp1gapcp2* (Fig. 3C). Analysis of GAPCp1 expression under the control of the *PHT* promoter indicated that the enzyme was located in leaf epidermal cell plastids (Fig. 4A), suggesting that it is necessary in these cell types. The expression analysis of the native *GAPCp1* promoter in GUS and GFP fusions corroborated that, in addition to its expression in vascular cells, the *GAPCp1* promoter directs gene expression in leaf epidermal cells (Fig. 4, B and C). The *PHT:GAPCp1* construct increased the root weight (Fig. 3C) by an increase of lateral root formation (Fig. 3E) but did not recover the growth of primary roots (Fig. 3D). This may be related to the lack of expression of *PHT:GAPCp1* in specific cell types of the root tip (Mudge et al., 2002). Analysis of the native GAPCp1 promoter in roots indicated that it was expressed in root vascular cells (Muñoz-Bertomeu et al., 2009), in epidermal cells, in the proximal part of meristems, in the columella, and in the lateral root cap cells (Fig. 4, B and C). *PHT:GAPCp1* started to be expressed

in the root epidermis at about 1.5 to 2 mm from the apex, having maximal expression at about 3 to 4 mm, but was not expressed in the meristem or the root cap (Fig. 4A). These results may suggest that GAPCp activity in root meristems is necessary for proper primary root growth.

The analysis of *gapcp1gapcp2 PHT:GAPCp1* lines confirmed that their metabolite profile in the AP is different from *gapcp1gapcp2* and similar to that of the wild type, according to Tukey's posthoc test of the ANOVA of the first principal component (Fig. 2E; Supplemental Table S3). Thus, in the *gapcp1gapcp2 PHT:GAPCp1* AP, 62% of metabolites (24 out of 39) were significantly changed as compared with *gapcp1gapcp2*, and most of them (14 out of 24) reached wild-type levels (Supplemental Table S3). In quantitative terms, the most important changes in *gapcp1gapcp2 PHT:GAPCp1* metabolites in relation to *gapcp1gapcp2* were the reductions in the levels of some amino acids (Asn and Gln) and sugars (Fru, maltose, Glc, and trehalose; Supplemental Table S3).
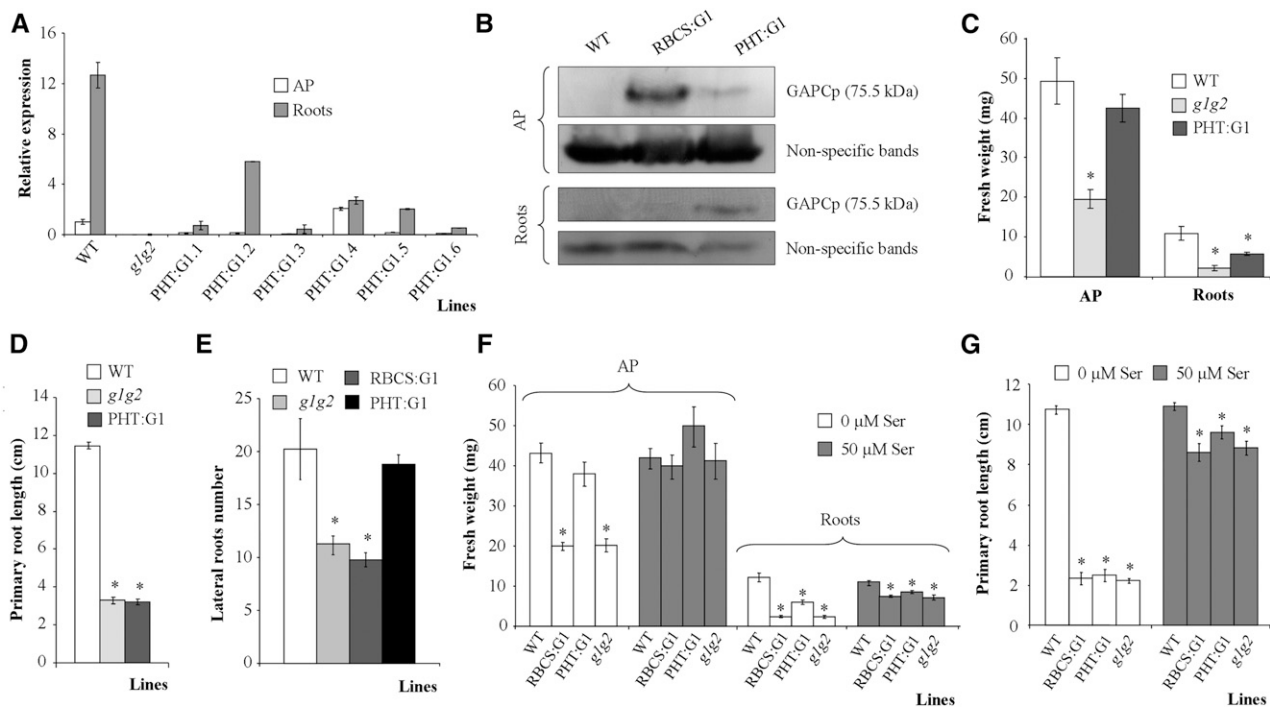
Figure 3. Molecular and phenotypical analyses of *gapcp1gapcp2* (*g1g2*) expressing GAPCp1-GFP under the control of the *PHT* promoter (PHT:G1). A, qRT-PCR analysis of *GAPCp1* in the APs and roots of several 21-d-old PHT:G1 lines as compared with *g1g2* and the wild type (WT). B, Immunoblot showing GAPCp1 expression in the APs and roots of representative RBCS:G1 and PHT:G1 lines as compared with the wild type. Protein gel-blot analysis was performed using anti-GFP antibodies. Nonspecific bands are shown as a sample loading control at bottom. C to E, Fresh weight (C), primary root length (D), and lateral root number (E) of 18-d-old wild-type, *g1g2*, and PHT:G1 plants. F and G, Fresh weight (F) and primary root growth (G) of wild-type, *g1g2*, RBCS:G1, and PHT:G1 lines in the absence or presence of 50 μM Ser. In A, values are means $\pm$ SE of three independent biological replications. In C to E, values are means $\pm$ SE of two independent transgenic lines ($n \geq 36$ plants). In F and G, values are means $\pm$ SE ($n \geq 40$ plants). Asterisks indicate significant differences as compared with the wild type ($P < 0.05$).

In roots, the metabolite profile of *gapcp1gapcp2 PHT:GAPCp1* was different from that of the wild type but also from that of *gapcp1gapcp2* and *gapcp1gapcp2 RBCS:GAPCp1*, according to Tukey's posthoc test of the ANOVA of the first principal component (Fig. 2F). The relative levels of 68% of metabolites analyzed in these lines (25 out of 37 metabolites) were significantly different from those of *gapcp1gapcp2* mutants, but only six of them reached wild-type levels (Supplemental Table S3). The intermediate metabolite profile of *gapcp1-gapcp2 PHT:GAPCp1* between the wild type and *gapcp1gapcp2* indicates a partial complementation of the *gapcp1gapcp2* phenotype and may reflect the lack of recovery of primary root growth (Fig. 3D). We previously proposed that one of the main functions of GAPCp in roots is to supply the 3-PGA precursor of Ser through the so-called PPSB (Muñoz-Bertomeu et al., 2009; Cascales-Miñana et al., 2013). The primary root growth of the *gapcp1gapcp2* mutant was 21% of that of the wild type in the absence of Ser and 81% of that of the wild type in its presence in the growth medium (Fig. 3G). In addition, growth of the AP was completely reestablished in the presence of Ser (Fig. 3F). The root growth of *gapcp1gapcp2 RBCS:GAPCp1* lines was

similar to that of *gapcp1gapcp2* in the presence of Ser, and that of *gapcp1gapcp2 PHT:GAPCp1* roots was still significantly lower than that of the wild type (Fig. 3, F and G). As shown by PCA, the metabolite profile of the AP of Ser-grown *gapcp1gapcp2* plants overlaps that of the wild type (Fig. 2D). However, Tukey's posthoc test of the ANOVA of the first principal component indicates that *gapcp1gapcp2* grown in the presence of Ser can be grouped within both subsets, that of the wild type and that of *gapcp1gapcp2* grown in the absence of Ser. These results indicate that, although Ser restores part of the *gapcp1gapcp2* metabolite values that come close to those found in the wild type, some metabolite changes still occur compared with the controls (Supplemental Table S2). This suggests that GAPCp may have other functions in addition to providing substrates for Ser biosynthesis via the PPSB in specific cell types.

Metabolic and Transcriptomic Pathways Affected by GAPCp

To assess the metabolic pathways affected by GAPCp activity in the AP and in roots, we followed a metabolomic and transcriptomic approach. Lack of GAPCp expression
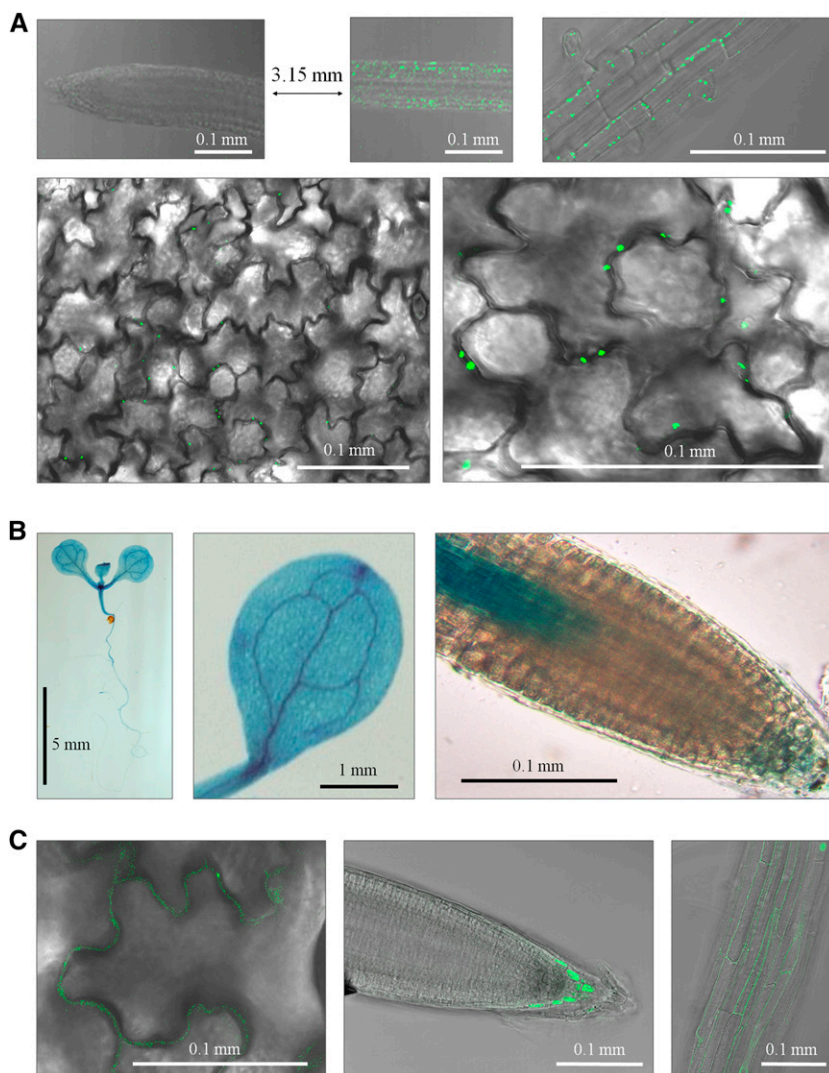
Figure 4. Cellular expression of GAPCp1 under the control of its native promoter or the *PHT* promoter. A, GAPCp1-GFP expression under the control of the *PHT* promoter at the root tip (top left), at 3.15 mm from the root tip (top middle and right), and in epidermal leaf cells (bottom). B, GUS expression under the control of the *GAPCp1* promoter (*ProG1:GUS*) in seedlings (left), cotyledons (middle), and root meristem (right). C, GFP expression under the control of the *GAPCp1* promoter (*ProG1:GFP*) in leaf epidermal cells (left), root columella (middle), and root epidermal cells (right).

clearly affected amino acid metabolism, as shown previously (Muñoz-Bertomeu et al., 2009), but also the carbon metabolism of sugars and organic acids (Tables I and II; Supplemental Tables S1–S3). Since metabolism is a dynamic process, we reasoned that a search for those metabolites that systematically changed under all different assayed conditions (end of the night, middle of the light period, end of the light period, and in the presence of Suc) may help narrow the identification of important pathways affected by GAPCp activity. We concentrated only on those metabolites that changed in *gapcp1gapcp2* independently of the growth conditions (Table I; Supplemental Tables S1–S4). In the AP, two amino acids related to Glu metabolism were increased, γ-aminobutyrate (GABA) and Gln. There was also an increase in many sugars and their derivatives (Fru, myoinositol, Fuc, galactinol, Glc, trehalose, and Xyl) in *gapcp1gapcp2* as compared with the wild type. However, the sugar phosphates Glc-6-P and Fru-6-P decreased in *gapcp1gapcp2* in all the experiments in which they could be quantified as compared with controls. Two organic

acids of the tricarboxylic acid cycle, citrate and succinate, also systematically increased in the AP of *gapcp1-gapcp2*, which suggests that the respiratory pathway is affected by the lack of GAPCp activity. Glycerate, a metabolite that can be synthesized from 3-PGA through the reaction catalyzed by 3-PGA phosphatase or, alternatively, used to synthesize 3-PGA through the reaction catalyzed by glycerate kinase, was also increased in *gapcp1gapcp2* when compared with the controls. In all instances, phosphoric acid was drastically reduced in *gapcp1gapcp2* (more than 90% under some conditions; Table I; Supplemental Tables S1–S3).

The 15 metabolites that systematically changed in the AP of *gapcp1gapcp2* all changed in the same direction in the *gapcp1gapcp2 RBCS:GAPCp1* lines (Table I). However, most of these changes were reverted in *35S: GAPCp1*, and some were reverted in *gapcp1gapcp2 PHT: GAPCp1* or *gapcp1gapcp2* grown in the presence of Ser. These results corroborate the lack of *gapcp1gapcp2* complementation when the enzyme is expressed in photosynthetic cells.

Table I. *List of metabolites that significantly increased (I) or decreased (D) in all assayed conditions in the AP of gapcp1gapcp2 (g1g2) when compared with wild-type plants*

Numerical values of *g1g2* (means ± SE) are relative values normalized to the mean response calculated for the wild type of one representative experiment. Significant changes (I or D) in *g1g2 RBCS:GAPCp1* (RBCS:G1), *g1g2 PHT:GAPCp1* (PHT:G1), and *g1g2 35S:GAPCp1* (35S:G1) lines as well as in *g1g2* plants grown in the presence of 100 μM Ser (*g1g2* Ser) when compared with the wild type are also reported. ND and NC stand for not determined and not changed, respectively. The full data sets are shown in Supplemental Tables S1 to S4.

Sample	g1g2	RBCS:G1	PHT:G1	35S:G1	g1g2 Ser
Metabolites					
Citrate	I (2.57 ± 0.23)	I	ND	NC	NC
Fru	I (2.71 ± 0.16)	I	I	I	I
Fru-6-P	D (0.86 ± 0.03)	D	D	ND	ND
Fuc	I (1.77 ± 0.10)	I	ND	NC	I
GABA	I (1.31 ± 0.06)	I	ND	D	I
Galactinol	I (8.86 ± 0.46)	I	D	D	NC
Glc	I (2.92 ± 0.13)	I	NC	NC	I
Glc-6-P	D (0.68 ± 0.06)	D	D	I	NC
Gln	I (1.94 ± 0.09)	I	NC	NC	I
Glycerate	I (3.18 ± 0.30)	I	NC	I	NC
Myoinositol	I (2.61 ± 0.13)	I	NC	NC	I
Phosphoric acid	D (0.13 ± 0.01)	D	D	NC	D
Succinate	I (1.20 ± 0.07)	I	ND	I	NC
Trehalose	I (1.47 ± 0.09)	I	I	NC	NC
Xyl	I (2.38 ± 0.13)	I	ND	I	I
No. of metabolites changed as compared with the wild type					
Changed in the same direction as *g1g2*	15	15	5	4	8
Not changed/opposite direction to *g1g2*	0	0	5	10	6
Not determined	0	0	5	1	1

In roots, only two different experiments were studied in either *gapcp1gapcp2* mutants or conditional *gapcp1-gapcp2* mutants (Supplemental Tables S3 and S4). In order to filter the results, we chose the metabolites that changed in both conditions and displayed more than a 1.5-fold change in at least one experiment (Table II). In general, amino acids tended to increase in *gapcp1-gapcp2*, but only Ala, Gln, Asn, and GABA fulfilled the chosen criterion. Regarding sugars, Fru, myoinositol, galactinol, raffinose, and trehalose increased more than

Table II. *List of metabolites that significantly increased (I) or decreased (D) in all assayed experiments in roots of gapcp1gapcp2 (g1g2), g1g2 RBCS: GAPCp1 (RBCS:G1), and g1g2 PHT: GAPCp1 (PHT:G1) when compared with wild-type plants*

Numerical values of *g1g2* (means ± SE) are relative values normalized to the mean response calculated for the wild type of one representative experiment. Significant changes in RBCS:G1 and PHT:G1 when compared with *g1g2* are also reported. Only metabolites that displayed more than a 1.5-fold change in at least one experiment are listed. NC stands for not changed. The full data sets are shown in Supplemental Tables S3 and S4.

Sample	Compared with the Wild Type			Compared with g1g2	
	g1g2	RBCS:G1	PHT:G1	RBCS:G1	PHT:G1
Metabolites					
Ala	I (1.94 ± 0.07)	I	I	NC	NC
Ala, β	I (3.05 ± 0.13)	I	I	NC	D
Asn	I (2.71 ± 0.29)	I	I	NC	D
Fru	I (2.38 ± 0.06)	I	I	I	D
GABA	I (1.86 ± 0.04)	I	I	NC	D
Galactinol	I (2.69 ± 0.07)	I	I	NC	D
Gln	I (1.91 ± 0.11)	I	I	NC	NC
Glycerate	I (3.89 ± 0.17)	I	I	I	D
Myoinositol	I (1.75 ± 0.07)	I	I	NC	D
Raffinose	I (1.87 ± 0.05)	I	I	I	NC
Threitol	D (0.20 ± 0.02)	D	D	NC	I
Trehalose	I (3.58 ± 0.16)	I	I	NC	D
No. of metabolites changed as compared with the wild type or *g1g2*					
Changed	12	12	12	3	9
Not changed	0	0	0	9	3

2-fold, while threitol decreased more than 50%, as compared with the controls. Glycerate increased more than 3-fold in *gapcp1gapcp2*. As in the AP, phosphoric acid was also depleted in roots of *gapcp1gapcp2*. In this case, the reduction was only about 40% (Supplemental Tables S3 and S4).

Changes in all these metabolites were maintained in *gapcp1gapcp2 RBCS:GAPCp1* lines as compared with the wild type (Table II). However, the levels of some of them (nine out of 12) were partly reverted in *gapcp1gapcp2 PHT:GAPCp1* and differed significantly from those in *gapcp1gapcp2*, which confirms the idea that GAPCp expression under the control of the *PHT* promoter partly complements *gapcp1gapcp2* (Table II). Seven out of 12 metabolites whose levels changed in *gapcp1gapcp2* roots (Fru, galactinol, glycerate, Gln, GABA, myoinositol, and trehalose) were also modified in the AP (Tables I and II), which suggest an effect of the *PHT:GAPCp1* construct in this organ.

A transcriptomic approach in the APs and roots showed that several functional categories of genes were up- or down-regulated in *gapcp1gapcp2* as compared with the wild type (Table III; Supplemental Table S5; Supplemental Materials S1). Changes in gene expression were also confirmed by qRT-PCR (Supplemental Fig. S3). According to the Munich Information Center for Protein Sequences Funcat Functional Annotation program (http://mips.gsf.de/proj/funcatDB; $P < 0.005$), only genes related to phosphate transport were significantly induced in the *gapcp1gapcp2* AP compared with the wild type (Table III). Specifically, two genes coding for phosphate transporters were up-regulated in the AP of *gapcp1gapcp2* (At2g38940 [*PHT1.4*] and At5g43370 [*PHT1.2*]; Supplemental Table S5). In order to further

specify important genes that might be affected by the lack of GAPCp activity, we compared misregulated genes in all microarrays obtained in this study and also with a previous microarray obtained by our group in which whole seedlings were analyzed (Muñoz-Bertomeu et al., 2009). We found that only two genes displayed common changes in all microarrays (wild-type versus *gapcp1-gapcp2* whole seedling, wild-type versus *gapcp1gapcp2* AP, wild-type versus *gapcp1gapcp2* roots, and *gapcp1-gapcp2* versus *gapcp1gapcp2 HS:GAPCp1* induced in APs and roots; see below), At5g06300 (*LONELY GUY7* [*LOG7*]) and At1g73010 (PYROPHOSPHATE-SPECIFIC PHOSPHATASE1 [*PPsPase1*]; Supplemental Table S5). Both genes were up-regulated in *gapcp1gapcp2* in all microarrays studied, and their induction was confirmed by qRT-PCR (Supplemental Fig. S3). Since PPsPase1 is postulated to play an important role in the inorganic phosphate (Pi) starvation response (May et al., 2011) and the metabolomic analysis of the AP of *gapcp1gapcp2* indicated a drastic reduction in the phosphoric acid level in the mutant, we searched in its transcriptome for additional misregulated genes involved in phosphate metabolism and homeostasis (Supplemental Table S5). In microarrays of *gapcp1gapcp2* AP, we found four up-regulated genes (At1g23140, At3g17790 [*PURPLE ACID PHOSPHATASE17*; *PAP17*], At5g20150, and At2g38940 [*PHT1.4*]) whose expression is also up-regulated in the shoots of the Ca^{2+}/H^+ transporter *cax1/cax3* mutants. The activity of this transporter is required for systemic phosphate homeostasis involving shoot-to-root signaling in Arabidopsis (Liu et al., 2011). Two additional genes, encoding a purple acid phosphatase (*PAP8*; At2g01890) and a phosphate transporter (*PHT1.2*; At5g43370), were also induced in the *gapcp1gapcp2* AP. Finally, from 16

Table III. *Functional categories of genes differentially expressed in the APs and roots of gapcp1gapcp2 as compared with the wild type*

Significantly enriched categories ($P < 0.005$) are shown.

Functional Category	P
APs	
Induced	
Phosphate transport	0.00441
Repressed	
Secondary metabolism	0.00457
Metabolism of glucosinolates and derivatives	0.00467
Stress response	0.00489
Roots	
Induced	
Nitrogen, sulfur, and selenium metabolism	0.00301
Nucleotide-sugar metabolism	0.00061
Lipid binding	0.00182
Cellular transport, transport facilities, and transport routes	0.00288
Transported compounds (substrates)	0.00369
Lipid/fatty acid transport	0.00110
Detoxification	0.00300
Oxygen and radical detoxification	0.00277
Response to biotic stimulus	0.00078
Repressed	
Monoterpene metabolism	0.00074

common misregulated genes in the APs and roots of *gapcp1gapcp2*, three were related to phosphate homeostasis. Apart from the previously mentioned *PPsPase1* (At1g73010), a second gene (At5g03545), known to be expressed in response to phosphate starvation, was induced in *gapcp1gapcp2* as compared with the controls. The third gene, At2g33770, encodes a ubiquitin-conjugating E2 enzyme, PHOSPHATE2, which was the only phosphorus-related gene repressed in both roots and AP of *gapcp1gapcp2*.

Since metabolomic and transcriptomic analyses indicated an alteration of Pi homeostasis, we measured the mineral content of *gapcp1gapcp2*. Several minerals were reduced in the *gapcp1gapcp2* AP, but the main nutrient deficiency observed was Pi (Supplemental Table S6). In *gapcp1gapcp2* roots, although some nutrients were reduced (potassium, copper, and iron), no Pi deficiency was observed, and some nutrients (nitrogen, manganese, and zinc) were even increased (Supplemental Table S6). In order to investigate whether mineral deficiency could affect *gapcp1gapcp2* development, we assayed the mutant growth in medium with increased mineral supply (Fig. 5). As compared with a one-fifth-strength Murashige and Skoog (MS) medium, growth in a one-half-strength MS medium only improved the AP growth in the *gapcp1gapcp2* PHT:GAPCp1 line, having no significant effect in the other lines and even decreasing root growth in the *PHT:GAPCp1* line. These results indicated that nutrient deficiency is not the main cause of the growth defects in *gapcp1gapcp2*.

Metabolite and Transcripts Responding to GAPCp Activity

In order to identify targets responding to GAPCp activity, we performed a metabolomic and transcriptomic study of conditional *gapcp1gapcp2* mutants. For this purpose, we used *gapcp1gapcp2* transformed with a *GAPCp1-GFP* cDNA under the control of the heat-shock promoter *HSP18.2* (*HS:GAPCp1*). Seven conditional lines were obtained, and two or three of them (depending on the experiment) were selected for further characterization, taking into account their basal *GAPCp1* level, their induction level, and their complementation of the *gapcp1gapcp2* root growth and sterile phenotype (Fig. 6; Supplemental Fig. S4). First, we performed a time-course experiment to decide the best time to perform the analyses. All selected *gapcp1gapcp2 HS:GAPCp1* lines displayed a peak of *GAPCp1* mRNA 1 h after induction (Fig. 6A). The GAPCp1 protein peak was reached 4 h after induction and was still present 8 h after induction (Fig. 6B; Supplemental Fig. S4). We assumed that translation is mostly resumed in Arabidopsis 1 h after a heat-shock treatment at 37°C (M. Castellanos, personal communication). Since GAPCp1 had to be translated and to participate in glycolysis before having an effect on transcription, we chose 4 h after induction for the realization of transcriptomic experiments and 8 h after induction for the metabolomic experiments. In order to discard any effect of heat shock
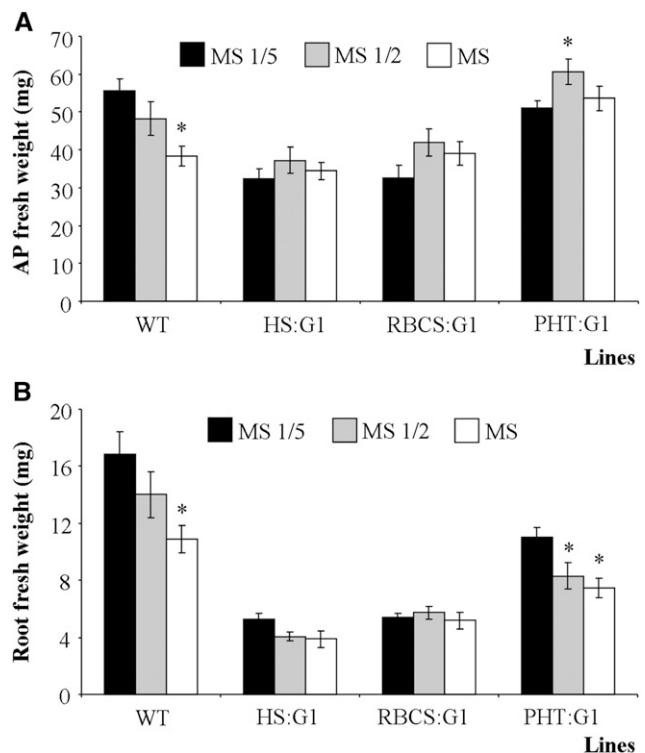

Figure 5. Growth parameters of *gapcp1gapcp2* lines grown in medium with increasing MS medium concentrations. Fresh weights of the AP (A) and roots (B) of 21-d-old wild type (WT) and *gapcp1gapcp2* plants expressing GAPCp1 (G1) under the control of the *HS* (HS:G1), the *RBCS* (RBCS:G1), and the *PHT* (PHT:G1) promoters are shown. Values are means ± se of two independent transgenic lines ($n \geq 36$ plants). Asterisks indicate significant differences as compared with plants grown in one-fifth-strength MS medium ($P < 0.05$).

on the metabolite profile of conditional mutants, we additionally studied the metabolite profile of *gapcp1-gapcp2* 8 h after a heat-shock treatment. Only one metabolite (Tyr) out of 42 measured changed in the AP, and four (Pro, Glc-6-P, Fru-6-P, and trehalose) out of 37 changed in the roots, of heat shock-treated *gapcp1gapcp2* as compared with untreated plants (Supplemental Table S7). Changes in these metabolites were not interpreted as such if the observed changes in the conditional mutants were in the same direction after induction.

The metabolomics analysis indicated that 54% (21 out of 39) and 22% (eight out of 37) of measured metabolites recovered wild-type levels in the APs and roots of induced *gapcp1gapcp2 HS:GAPCp1*, respectively (Supplemental Table S4). In general, lower amino acid and sugar levels were observed in these lines, which indicates the crucial role of GAPCp activity in nitrogen and carbon metabolism. Tables IV and V list the metabolites whose levels changed by more than 20% following GAPCp1 induction in APs and roots, respectively. Three of them, Gln, glycerate, and galactinol, are particularly interesting because they were the only ones altered in all experimental conditions assayed in both roots and AP of *gapcp1gapcp2* and those that displayed the most
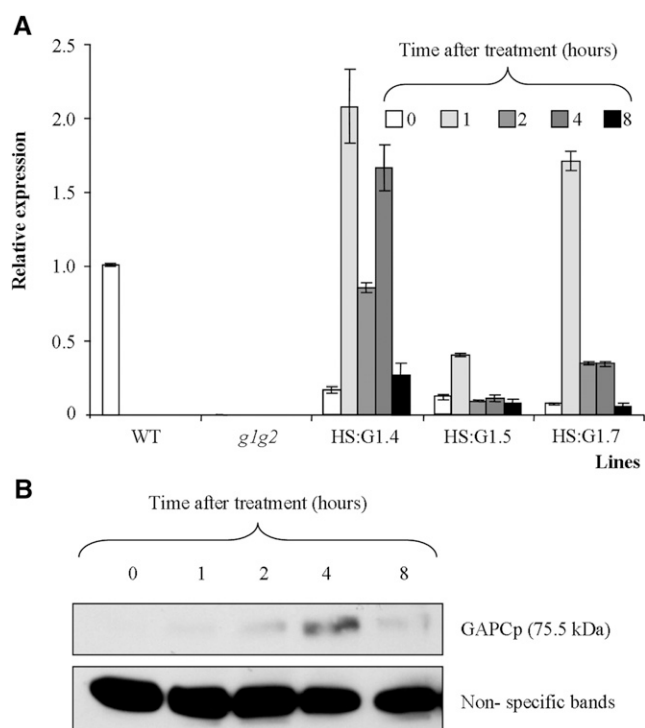
A

B

Figure 6. Molecular and phenotypical analyses of *gapcp1gapcp2* (*g1g2*) expressing GAPCp1-GFP (G1) under the control of the *HS* promoter (HS:G1). A, Time-course analysis of *GAPCp1* mRNA expression in three 18-d-old HS:G1 lines as compared with the wild type (WT) and *g1g2*. B, Time-course analysis of GAPCp1 protein expression in HS:G1 lines. Protein gel-blot analysis was performed using anti-GFP antibodies. Nonspecific bands are shown as a sample loading control at bottom. In A, values means ± SE of three independent biological replications.

important changes after GAPCp1 induction. In the AP, Gln was the metabolite whose levels changed the most after GAPCp1 induction (31% reduction). Galactinol and glycerate were reduced by 24% and 22%, respectively. In roots, the levels of these three metabolites lowered even more drastically than in the AP after GAPCp1 induction, with glycerate reduced the most of all metabolites measured (68% reduction), followed by Gln (45%) and galactinol (33%). To provide additional insights that could explain changes in glycerate levels, we next measured the activity of phosphoglycerate kinase in roots and in the AP. The enzyme activity was higher in the AP of *gapcp1gapcp2* than in the wild type (264 ± 6 versus 246 ± 2 nmol NADH g^{-1} fresh weight min^{-1} in *gapcp1-gapcp2* and the wild type, respectively) and especially in roots (60 ± 2 versus 34 ± 2 nmol NADH g^{-1} fresh weight min^{-1} in *gapcp1gapcp2* and the wild type, respectively), where the highest levels of glycerate in the mutant were found.

The high levels of Gln in *gapcp1gapcp2* could be related to the arrest in PPSB activity, which has been shown to affect the ammonium assimilation pathway (Benstein et al., 2013). According to this, the ammonium content in *gapcp1gapcp2* roots was significantly higher

than in the wild type (Fig. 7), which could contribute to growth impairment in the mutant. Ammonium toxicity is currently associated with acidification of the medium (Li et al., 2014). To study such acidification, we used the visual assay for H$^+$ efflux from root cells based on plates containing the pH indicator Bromocresol Purple. As indicated in Figure 7, the yellow color surrounding the roots of *gapcp1gapcp2* roots, indicative of moderate acidification, was much greater than in the wild type.

The transcriptomic study revealed that, in the AP, several functional categories of genes were significantly up- or down-regulated following the induction of GAPCp1 (Supplemental Table S8). In order to narrow the identification of putative targets responding to GAPCp1 activity, we searched for those genes that were altered in *gapcp1gapcp2* as compared with the wild type and whose expression was reverted by the induction of GAPCp1 activity in the mutant. We considered that these genes could be mediating the response to altered GAPCp1 activity. Twenty-four genes in the AP and three genes in roots whose expression was misregulated in *gapcp1gapcp2* were then reverted after the induction of GAPCp1 (Table VI). Hormone (At5g06300, At1g67100, At1g80340, At3g23150, and At4g37150) and sugar signaling (At3g30720), cell cycle regulation (At2g29680), and metabolism (At5g17330, At5g52570, and At5g67520) associated genes fitted these criteria (Table VI). Two of them are particularly interesting (At5g06300 [*LOG7*] and At5g17330, which encodes one of two isoforms of Glu decarboxylase [*GAD*]) as development and metabolic mediators of the response to altered GAPCp1 activity. *LOG7*, one of the two common misregulated genes in all *gapcp1gapcp2* microarrays, was up-regulated in *gapcp1gapcp2* APs and roots and down-regulated in both organs upon GAPCp1 induction. *LOG7*, which encodes an enzyme participating in cytokinin biosynthesis, has been implicated in the maintenance of the shoot apical meristem and plays a role in normal primary root growth (Tokunaga et al., 2012). Similarly, *GAD* was up-regulated in the *gapcp1gapcp2* AP and down-regulated after GAPCp1 activity induction. This enzyme, which

Table IV. *Metabolites in AP of gapcp1gapcp2 HS:GAPCp1 plants that significantly changed more than 20% following GAPCp1 induction*

Data for *gapcp1gapcp2 HS:GAPCp1* induced and noninduced plants (means ± SE) are relative values normalized to the mean response calculated for wild-type plants. The full data set is shown in Supplemental Table S4.

Metabolite	Noninduced	Induced
Arg	1.01 ± 0.06	0.80 ± 0.05
Galactinol	1.19 ± 0.05	0.91 ± 0.04
Glc	1.38 ± 0.07	1.05 ± 0.07
Gln	1.53 ± 0.11	1.05 ± 0.08
Glycerate	1.44 ± 0.04	1.13 ± 0.06
Gly	0.79 ± 0.03	0.98 ± 0.03
Maltose	0.92 ± 0.08	1.33 ± 0.04
Tyr	1.04 ± 0.11	0.56 ± 0.05
Urea	0.66 ± 0.03	0.94 ± 0.03

Table V. *Metabolites in roots of gapcp1gapcp2 HS:GAPCp1 plants that significantly changed more than 20% following GAPCp1 induction*

Data for *gapcp1gapcp2 HS:GAPCp1* induced and noninduced plants (means ± SE) are relative values normalized to the mean response calculated for wild-type plants. The whole metabolite set is presented in Supplemental Table S4.

Metabolite	Noninduced	Induced
Ala	2.01 ± 0.20	1.47 ± 0.11
Ala, β	2.33 ± 0.15	1.43 ± 0.10
Asn	2.79 ± 0.40	1.82 ± 0.21
Asp	1.35 ± 0.06	0.88 ± 0.01
Fru-6-P	0.99 ± 0.05	0.72 ± 0.03
Galactinol	2.25 ± 0.15	1.50 ± 0.07
Glc-6-P	0.96 ± 0.05	0.65 ± 0.02
Glu	1.17 ± 0.09	0.84 ± 0.05
Gln	2.30 ± 0.23	1.26 ± 0.12
Glycerate	4.40 ± 0.06	1.42 ± 0.07
Myoinositol	1.52 ± 0.07	1.21 ± 0.05
Malate	1.18 ± 0.04	0.92 ± 0.03
Raffinose	1.96 ± 0.13	1.51 ± 0.08
Trehalose	2.29 ± 0.20	1.71 ± 0.12

plays an important role in balancing carbon and nitrogen metabolism, catalyzes the irreversible decarboxylation of Glu to form GABA (Fait et al., 2011).

DISCUSSION

GAPCp Activity in Nonphotosynthetic Cells Restores the Metabolite Profile and Growth of the AP

Knowledge concerning the specific roles of glycolysis in photosynthetic and heterotrophic cells is scarce. Given that *gapcp1gapcp2* displays a drastic growth reduction of the AP, we explored the specific effect of GAPCp in photosynthetic cells by expressing the enzyme under the control of the *RBCS* promoter. Morphological and metabolomic analysis indicated that *RBCS:GAPCp1* expression did not complement the reduced growth phenotype either in roots or in the AP. The metabolite profile of the AP in *gapcp1gapcp2* and *gapcp1gapcp2 RBCS:GAPCp1* was similar under both photosynthetic and nonphotosynthetic conditions. These results indicate that plastidial glycolysis, or at least GAPCp activity, is not functionally important in photosynthetic cells. GAPCp1 expression under the control of the *35S* promoter, however, restored both the growth phenotype and the metabolite profile of *gapcp1gapcp2*, suggesting that metabolic alterations in the AP could be caused by a lack of GAPCp activity in roots or in other AP cell types. In this sense, GAPCp1 expression under the control of the *PHT* promoter improved the growth of the AP in *gapcp1gapcp2* and modified its metabolite profile. We found that *PHT1.2* was not only expressed in roots, as described elsewhere (Mudge et al., 2002; Nussaume et al., 2011; Kirchsteiger et al., 2012), but also in the leaf epidermal cells of *gapcp1gapcp2*. *PHT:GAPCp1* expression in the AP may

be related to the Pi-deficient phenotype of *gapcp1-gapcp2*, as this promoter is induced by Pi starvation in wild-type roots (Mudge et al., 2002), although not in the AP, as was observed in *gapcp1gapcp2*. Given that GAPCp1 expression in photosynthetic cells did not complement *gapcp1gapcp2* AP phenotypes, we postulate that improved AP metabolism and development by *PHT:GAPCp1* expression could be the result of GAPCp expression in AP heterotrophic cells, such as epidermal or vascular cells, where GAPCp is currently expressed under the control of its native promoter. This would explain why most metabolites whose levels changed in *gapcp1gapcp2* roots were also modified in the AP, why some of the metabolites that directly respond to GAPCp activity, such as Gln and glycerate, increased in both *gapcp1gapcp2* APs and roots, and why these metabolites reached wild-type levels in the *gapcp1gapcp2 PHT:GAPCp1* AP. However, an indirect effect of GAPCp1 expression in roots on AP metabolism cannot completely be ruled out.

The partial complementation of *gapcp1gapcp2* when expressing *PHT:GAPCp1* may be due to the enzyme not being present in specific root cell types. Confocal microscopy indicated that GAPCp1 expression under the control of the *PHT* promoter was observed in root
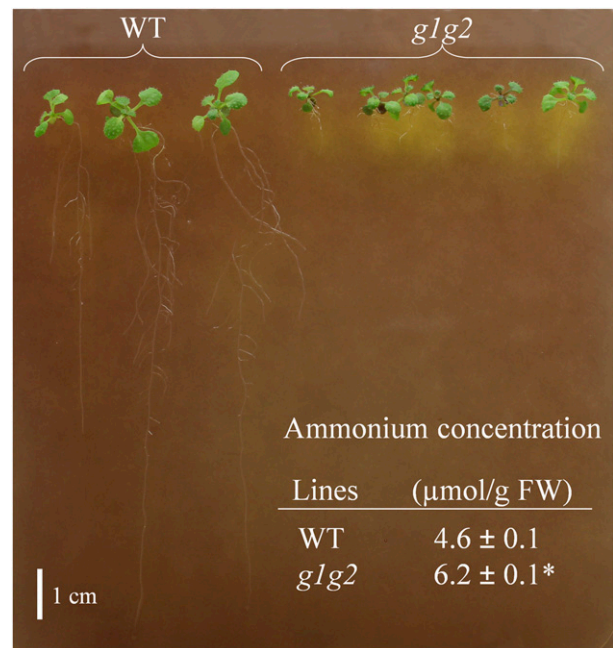
Figure 7. Ammonium content and proton extrusion increase in roots of *gapcp1gapcp2*. Two-week-old Arabidopsis *gapcp1gapcp2* (*g1g2*) and wild-type (WT) plants were grown for 6 h in one-fifth-strength MS medium at pH 5.7 without MES and containing 0.003% (w/v) Bromocresol Purple. The yellow color of the pH indicator around *g1g2* roots indicates increased proton extrusion in the mutant. The table at bottom presents data of ammonium content (means ± SE; *n* = 3) in roots of *g1g2* and the wild type. The asterisk indicates a significant difference as compared with the wild type ($P < 0.05$). FW, Fresh weight.

Table VI. *List of genes in APs and roots whose expression was misregulated in gapcp1gapcp2 (g1g2) as compared with the wild-type plants and then reverted after GAPCp1 induction in g1g2 HS:GAPCp1 (HS:G1)*

Gene	Fold Change (Log 2)		Gene Description
	Wild Type versus *g1g2*	*g1g2* versus HS:G1	
APs			
At1g14880	−2.6	1.7	Plant Cadmium Resistance1 (PCR1)
At1g23840	−1.4	1.3	Unknown function
At1g53480	−2.0	1.6	Encodes MRD1 (mto1 responding down)
At1g67100	−1.9	0.9	LOB domain-containing protein40 (LBD40)
At3g23150	−0.8	1.0	Involved in ethylene perception in Arabidopsis
At3g23440	−1.3	0.7	Embryo Sac Development Arrest6 (EDA6)
At3g30720	−0.6	1.0	Qua-Quine Starch (QQS)
At4g13345	−0.9	2.3	Maternal Effect Embryo Arrest55 (MEE55)
At4g22280	−0.8	0.9	F-box/RNI-like superfamily protein
At5g13270	−0.6	0.8	Encodes RARE1 (Required for accD RNA Editing1)
At5g41620	−0.6	1.9	Unknown function
At5g67520	−1.5	0.7	Provides activated sulfate for the sulfation of secondary metabolites, including the glucosinolates
At1g59930	2.1	−1.1	Encodes a maternally expressed imprinted gene
At1g80090	2.5	−1.4	Cystathionine β-synthase (CBS) family protein
At1g80340	1.0	−0.9	Encodes a protein with GA$_3$ β-hydroxylase activity
At2g01890	1.0	−1.1	Encodes a purple acid phosphatase (PAP)
At2g29680	1.4	−0.7	Encodes cell division control protein6 (CDC6)
At3g11580	2.5	−0.6	APETALA2/B3-like transcriptional factor family protein
At3g59700	1.7	−0.7	Member of a receptor kinase-like protein family
At4g01985	0.6	−0.9	Unknown function
At4g37150	1.2	−1.1	Methyl Esterase9 (MES9)
At5g06300	3.0	−1.3	Putative Lys decarboxylase family protein (LOG7)
At5g17330	0.7	−0.8	Encodes one of two isoforms of Glu decarboxylase (GAD1)
At5g52570	1.3	−0.9	Converts β-carotene to zeaxanthin via cryptoxanthin (BCH2)
Roots			
At1g78070	−1.2	0.7	Transducin/WD40 repeat-like superfamily protein
At5g03090	−4.5	1.8	Unknown function
At5g06300	1.4	−2.5	Putative Lys decarboxylase family protein (LOG7)

differentiated cells but not in meristematic and root cap cells, where GAPCp (this work) and PPSB genes are normally expressed (Cascales-Miñana et al., 2013; Toujani et al., 2013). It has been postulated that PPSB is an important source of Ser in specific cell types, such as the anther tapetum and meristematic cells (Ros et al., 2014). Lack of expression of *PHT:GAPCp1* in *gapcp1-gapcp2* root meristematic cells and root cap cells would prevent the supply of Ser to these cells and thus prevent the recovery of primary root growth. This idea is supported by the recovery of *gapcp1gapcp2* primary root growth by Ser supplementation. Regarding this observation, it would be interesting to know why the AP growth is reduced in *gapcp1gapcp2*, since this organ is not Ser deficient (Muñoz-Bertomeu et al., 2009; this work). It could be that the AP growth inhibition is due to mineral deficiency, and specifically Pi deficiency, as a consequence of root growth inhibition in the mutant. Organic and mineral Pi deficiency was measured in the AP of *gapcp1gapcp2*. However, an increased supply of mineral nutrients did not significantly increase the AP growth or the primary root growth of *gapcp1gapcp2* or *gapcp1gapcp2 RBCS:GAPCp1* lines. Besides, GAPCp1 expression in roots directed by the *PHT* promoter did not increase organic or mineral Pi content but

significantly improved AP growth. Thus, Pi deficiency is probably not the primary cause of AP growth inhibition in *gapcp1gapcp2*. However, it remains possible that this deficiency could potentiate the phenotypic alterations observed in the mutant.

Lack of GAPCp Activity Affects Nitrogen and Carbon Metabolism and Mineral Nutrition

Several genes coding for proteins involved in Pi transport and signaling were up-regulated in the AP of *gapcp1gapcp2*. This observation is in accordance with the Pi deficiencies observed in the *gapcp1gapcp2* AP, which could limit its growth. PPsPase1 deserves a special mention, since it was commonly induced in all the *gapcp1gapcp2* microarrays. *PPsPase1* encodes a pyrophosphate-specific phosphatase (PPsPase1) that has been suggested to play a role in the Pi starvation response by providing Pi from phosphorylated substrates (May et al., 2011). However, the expression pattern of eight out of nine misregulated Pi-related genes in *gapcp1gapcp2*, including PPsPase1, was not modified by GAPCp1 activation, which indicates that they are not genes primarily responding to GAPCp1 induction. This would corroborate that Pi deficiency is

the consequence rather than the cause of the inhibition of primary root growth in *gapcp1gapcp2* mutants.

In roots, misregulated functional categories included nitrogen and sulfur metabolism and transport of amino acids and ions. Unlike in the AP, genes involved in stress responses were induced in *gapcp1gapcp2* roots, although in both roots and the AP metabolite markers related to stress (raffinose and galactinol) were increased. The metabolomic analysis also indicated that not only carbon metabolism, including the tricarboxylic

acid cycle, but also amino acid metabolism was seriously affected in *gapcp1gapcp2*. These results indicate that GAPCp is required to maintain the normal interactions between carbon and nitrogen metabolism that, in turn, affect plant development. The dramatic phenotypic and metabolic alterations in GAPCp mutants, therefore, may be related to multiple requirements of the products of GAPCp in plant metabolism rather than to a specific role in glycolysis itself. Indeed, the effects of a lack of GAPCp activity in the metabolite profile were
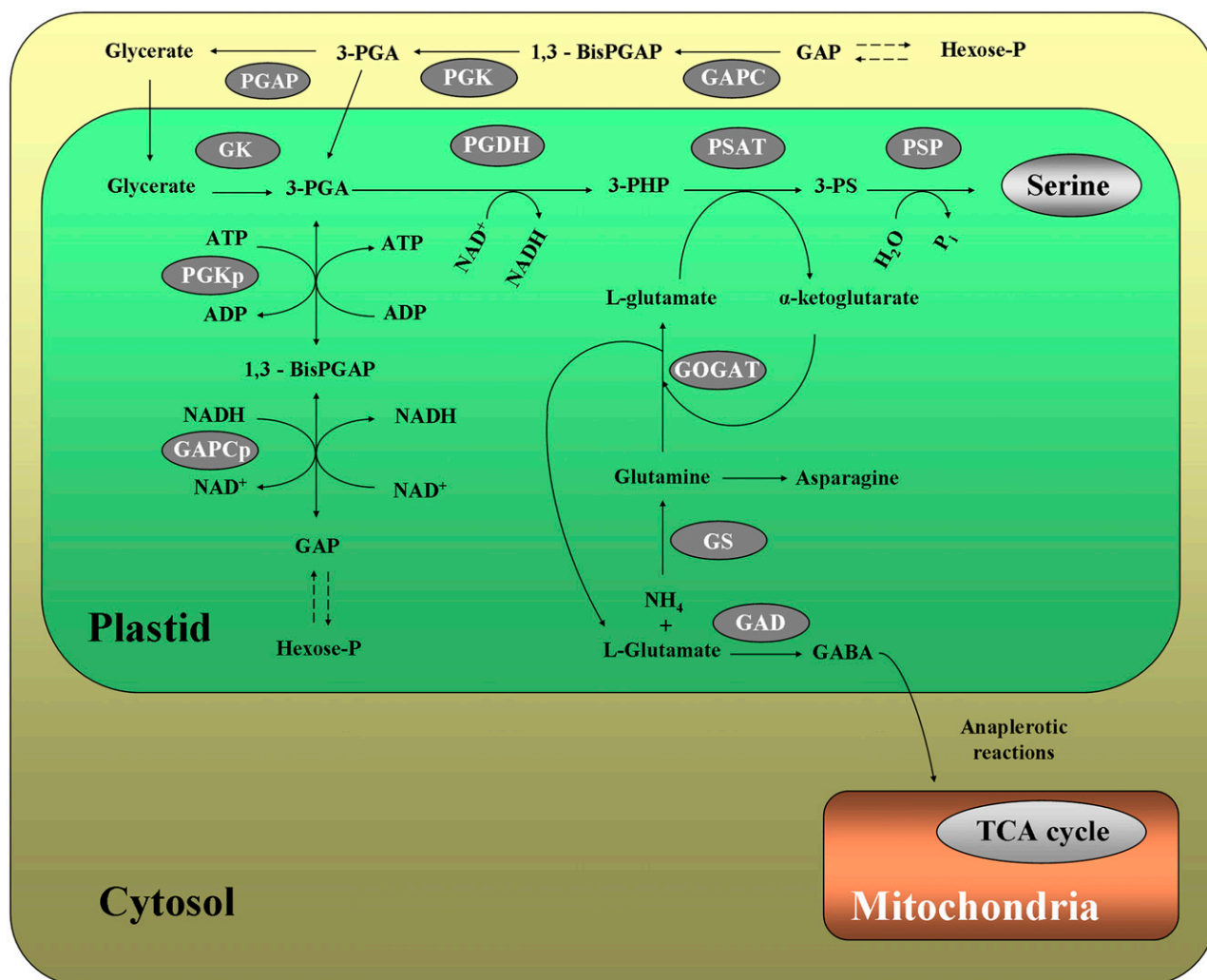

Figure 8. Proposed model for metabolic pathways affected by GAPCp activity. Lack of GAPCp activity reduces the 3-PGA precursor for the PPSB, which provides Ser to specific nonphotosynthetic cells but also α-ketoglutarate required for ammonium assimilation through the Gln synthetase/Glu synthase (GS/GOGAT) pathway and/or for anaplerotic reactions into the tricarboxylic acid (TCA) cycle. The reduced activity of the GS/GOGAT pathway would increase Gln and would divert L-Glu to the biosynthesis of GABA, which could play a role in balancing carbon and nitrogen metabolism in *gapcp1gapcp2* by contributing to the anaplerotic flux of Gln-derived carbon into the tricarboxylic acid cycle when PPSB is restricted. The reduced 3-PGA levels in plastids could be compensated in part by the transport of glycerate from the cytosol and subsequent conversion into 3-PGA by glycerate kinase (GK). The enzymes participating in each biosynthetic pathway are as follows: for the phosphorylated pathway (PPSB), 3-phosphoglycerate dehydrogenase (PGDH), 3-phospho-Ser aminotransferase (PSAT), and 3-phospho-Ser phosphatase (PSP); and for the ammonium assimilation pathway, GS/GOGAT. Other enzymes are as follows: PGAP, phosphoglycerate phosphatase; and PGK, phosphoglycerate kinase; p stands for plastidial. Metabolites are as follows: 1,3-bis-PGAP, 1,3-bis-phosphoglycerate; GAP, glyceraldehyde 3-phosphate; 3-PHP, 3-phosphohydroxypyruvate; and 3-PS, 3-phospho-Ser.

much less severe when plants were grown in the presence of Ser, which partially rescues primary root growth, indicating that the main role of GAPCp could be the supply of precursors for PPSB rather than its role in glycolysis per se.

Metabolite and Gene Targets of GAPCp Activity

Three metabolites (galactinol, glycerate, and Gln) systematically changed in both roots and the AP of *gapcp1gapcp2* and tend to recover wild-type levels upon GAPCp1 induction. Galactinol (and myoinositol) increased in *gapcp1gapcp2* and decreased after GAPCp induction in conditional mutants. Raffinose also increased in *gapcp1gapcp2* in most of the assayed experimental conditions. These metabolites participate as substrates (galactinol and myoinositol) or final products (raffinose) in the biosynthesis of the raffinose family oligosaccharides (Nishizawa et al., 2008) and have been shown to play a role in the plant response to environmental stresses (Taji et al., 2002; Kaplan et al., 2007). It has been postulated that galactinol and raffinose could increase tolerance to chilling, salinity, or drought stress by protecting plants against oxidative damage (Nishizawa et al., 2008). The high level of these metabolites in *gapcp1gapcp2* is probably an indicator of oxidative stress in *gapcp1gapcp2*. This could be a secondary consequence of metabolic perturbations resulting from the lack of GAPCp. Alternatively, GAPCp could be involved in plant protection against oxidative stress, a nonmetabolic function already suggested for the plant cytosolic GAPCs (Baek et al., 2008; Guo et al., 2012).

Glycerate accumulated in *gapcp1gapcp2*, especially in roots, where the levels clearly lowered after GAPCp1 activation in conditional mutants. Glycerate can be converted into 3-PGA in a reaction catalyzed by glycerate kinase in plastids (Fig. 8). Kleczkowski and Randall (1986) proposed that glycerate kinase, in addition to its role in photorespiration in leaves, could serve in C_4 species as part of a facilitated diffusion system for the intercellular transport of 3-PGA. Glycerate accumulation in *gapcp1gapcp2* roots may form part of the plant response to maintain 3-PGA levels in plastids. The high phosphoglycerate kinase activity measured in *gapcp1-gapcp2* would be anticipated to provide an increased 3-PGA concentration that would be converted into glycerate by the phosphoglycerate phosphatase (Fig. 8). If this is correct, the increased glycerate concentration in *gapcp1gapcp2* should be related to the activity of the cytosolic phosphoglycerate kinase isoform, since the activity of the plastidial isoform should be compromised in the mutant due to the lack of substrate provided by GAPCp in vivo. Glycerate accumulated in the cytosol could then be transported into the plastid, where glycerate kinase would convert glycerate into 3-PGA (Fig. 8). A glycerate transporter was recently identified in Arabidopsis chloroplasts (Pick et al., 2013). However, both glycerate and the triose-phosphate

transporters, which can efficiently exchange 3-PGA and glycerate between the cytosol and the plastid, are poorly expressed or nonexpressed in roots (http://www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) and probably in nonphotosynthetic cells in general. We propose that the absence of GAPCp constrains plastid 3-PGA production, making plastids dependent on a cytosolic source of this precursor. This could be supplied either as 3-PGA or glycerate. Since the capacities of both Triose Phosphate Translocator and glycerate transporter are likely to be limited in nonphotosynthetic cells, 3-PGA may be imported via the Glc-P/Pi translocator (albeit inefficiently); alternatively, glycerate, which is present at high levels in *gapcp1gapcp2*, may also enter by diffusion. If the latter, then plastidic glycerate kinase activity could be a limiting factor.

Gln levels were also significantly altered in both roots and the AP following the induction of the enzyme in conditional mutants. The connection between GAPCp, PPSB, and the ammonium assimilation pathway (GS/GOGAT; Muñoz-Bertomeu et al., 2009; Benstein et al., 2013; Cascales-Miñana et al., 2013) could provide clues about the possible mechanism involved in the modification of Gln content. The second enzyme of the PPSB, PSAT, uses L-Glu as cosubstrate in the formation of α-ketoglutarate (Fig. 8). If PSAT activity is restricted, α-ketoglutarate would not be regenerated, and then the second reaction of the GS/GOGAT pathway (GOGAT), which uses α-ketoglutarate as cosubstrate along with Gln, would be inhibited. Thus, Gln levels would increase in the cell. Consequently, the high levels of Gln found in the roots and the AP of *gapcp1gapcp2* may be a direct consequence of the arrest in PPSB activity, which in turn affects the GS/GOGAT pathway. The facts that NH_4^+ levels are increased in roots of *gapcp1gapcp2* and that one of the main changes of activating GAPCp1 was the reduction of Gln levels in both roots and the AP would support this idea. In addition, levels of Gln were not reduced in the AP of *gapcp1gapcp2* supplied with Ser when the PPSB would be anticipated to be inactive. This would support the high Gln levels in the mutant coming from the reduction of PSAT activity. Gln is the primary product of nitrogen assimilation in plants and a central metabolite of nitrogen metabolism. Although as yet unknown, a Gln-sensing mechanism similar to that recently identified in other plant families (Chellamuthu et al., 2014) could play a pivotal role in coordinating nitrogen metabolism in response to the general metabolic state of the cell. In this regard, Gln along with Asn are the main organic nitrogen forms transported between roots and the AP. Indeed, Gln is the most abundant amino acid in the xylem and phloem sap (Zhang et al., 2010). As such, Gln could be a good candidate for communication between the root and the AP in order to induce the required metabolic adjustment in *gapcp1gapcp2*.

Concerning gene targets responding to altered GAPCp1 activity, many functional categories were significantly affected by the induction of the enzyme. Many hormone-signaling genes (including those involved in

cytokinin, GA, ethylene, and jasmonate responses) responded to the activation of the enzyme, which suggests that they participate in the recovery of plant development. One of them, known as *LOG7*, is a Lys decarboxylase (cytokinin riboside 5'-monophosphate phosphoribohydrolase) that was up-regulated in both the APs and roots of *gapcp1gapcp2* and down-regulated upon GAPCp1 induction. LOG7 has been described as performing a regulatory function in the shoot and root apical meristem and could play an important role in the control of plant development through the cytokinin activation pathway (Tokunaga et al., 2012). Transfer DNA (T-DNA) insertion mutants in which the LOG-dependent pathway is impaired displayed severe retardation of shoot and root growth (Tokunaga et al., 2012). We found that *LOG7* was not repressed, but rather induced, in *gapcp1gapcp2*, which may suggest that *LOG7* up-regulation is a consequence of the mutant's reduced growth rather than a cause of it.

Another gene candidate that could mediate the lack of GAPCp activity is At5g17330, which codes for one of the isoforms of GAD. This enzyme participates in Glu metabolism by catalyzing its irreversible decarboxylation to form GABA (Fait et al., 2011; Fig. 8). The increased GABA levels measured in roots, and especially in the AP, of the *gapcp1gapcp2* mutant could well be the consequence of a higher GAD expression in the mutant. GAD could metabolize L-Glu to avoid the accumulation of Gln through the GS reaction. In this sense, GAD expression is repressed after GAPCp1 activation and Gln levels are lowered. On the other hand, GABA catabolism produces succinic semialdehyde, which is converted into succinate by succinic semialdehyde dehydrogenase. We can speculate that this mechanism might contribute to the anaplerotic flux of Gln-derived carbon into the tricarboxylic acid cycle. Increased GAD activity could form part of a metabolic adjustment in *gapcp1gapcp2* to restore metabolite homeostasis. Fait et al. (2011) proposed that GAD-mediated conversion of L-Glu into GABA plays a role in balancing carbon and nitrogen metabolism. In this vein, overexpression of GAD during seed maturation increases the nitrogen-to-carbon ratio. As *gapcp1gapcp2* mutant roots have an increased nitrogen-to-carbon ratio (Muñoz-Bertomeu et al., 2009), GAD could be a mediator of the response to altered GAPCp1 activity connecting carbon and nitrogen metabolism. We propose a model in which a lack of GAPCp1 activity reduces the PPSB flux that provides Ser to specific heterotrophic cells but also supplies α-ketoglutarate required for the GS/GOGAT pathway (Fig. 8). Inhibition of the GS/GOGAT pathway would increase Gln and divert L-Glu to the biosynthesis of GABA. This metabolite may play a role in balancing carbon and nitrogen metabolism in *gapcp1gapcp2* by contributing to the anaplerotic flux of Gln-derived carbon into the tricarboxylic acid cycle (Fig. 8). The 3-PGA deficit in the plastids of specific heterotrophic cells, therefore, would be compensated by increased glycerate and 3-PGA biosynthesis in the cytosol, which we believe would be inefficiently transported to the plastid lumen. Further studies are needed, however, to fully understand how these metabolic and transcriptomic networks are connected and how they interact through GAPCp to regulate plant metabolism and development.

CONCLUSION

The results presented here show that GAPCps do not have a major function in photosynthetic cells but play a crucial role in nonphotosynthetic cells of both photosynthetic and nonphotosynthetic organs, where they could provide essential metabolites for plant survival. The very specific expression pattern of GAPCp in primary root meristems and the root cap could be the key for its important role in this organ. Our results indicate that GAPCp interacts with essential metabolic pathways that affect nitrogen and carbon metabolism and in turn interfere with plant development. Then, GAPCp seems to be an important metabolic connector of essential pathways of primary metabolism in plants. A prerequisite of metabolic engineering is the precise understanding of plant metabolic networks and how they are interconnected. The results here open new directions for research on the mechanisms connecting plant metabolic networks.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) initial seed stocks (ecotype Columbia-0) were supplied by the European Arabidopsis Stock Centre (Scholl et al., 2000). The plastidial *gapcp1gapcp2* is a T-DNA insertional double mutant (SAIL_390_G10 and SALK_137288) of genes At1g79530 and At1g16300, respectively, which was obtained previously by our group (Muñoz-Bertomeu et al., 2009). Unless stated otherwise, seeds were vertically sown on 0.8% (w/v) agar plates containing one-fifth-strength MS medium with Gamborg vitamins, as described previously (Muñoz-Bertomeu et al., 2009). Some plates were supplemented with Ser, additional MS salts, or 0.003% (w/v) Bromocresol Purple as indicated in the figure legends. For acidification studies, plates were prepared without MES and adjusted to pH 5.7 with KOH. For the selection of transgenic plants, one-half-strength MS plates supplemented with 0.5% (w/v) Suc and appropriate selection markers were used. Some plantlets were also grown under greenhouse conditions as described elsewhere (Muñoz-Bertomeu et al., 2009). Wherever indicated, conditional *gapcp1gapcp2* was treated for 1 h at 37°C several hours before sampling.

Primers

All primers used in this work are listed in Supplemental Table S9.

Cloning and Plant Transformation

The cDNA corresponding to *GAPCp1* (At1g79530) was placed under the control of four different promoters: 35S promoter, AP-specific promoter of gene At5g38420 (RBCS2B; Kim et al., 2005), promoter of gene At5g43370 (PHT1.2; Mudge et al., 2002), and heat shock-inducible promoter of gene At5g59720 (HSP18.2; Matsuhara et al., 2000), giving constructs *35S:GAPCp1*, *RBCS:GAPCp1*, *PHT:GAPCp1*, and *HS:GAPCp1*, respectively.

Construct *35S:GAPCp1* carrying GAPCp1 fused in frame with GFP at the C-terminal position (GAPCp1-GFP) in plasmid pMDC83 (Curtis and Grossniklaus, 2003) was obtained previously as described (Muñoz-Bertomeu et al., 2009). This plasmid was used to obtain constructs *RBCS:GAPCp1*, *PHT:GAPCp1*,

and *HS:GAPCp1*. The *35S* promoter of *35S:GAPCp1* was exchanged with the 1,335-bp *RBCS* promoter previously PCR amplified from plasmid pSBright (Kim et al., 2005), kindly provided by Dr. D. Alabadí (Instituto de Biología Molecular y Celular de Plantas-Consejo Superior de Investigaciones Científicas), using primers described in Supplemental Table S9 to introduce *Hin*dIII and *Pac*I sites, giving *RBCS:GAPCp1*. For construct *PHT:GAPCp1*, the *35S* promoter of *35S:GAPCp1* was exchanged with the 2,012-bp *PHT* promoter previously PCR amplified from plasmid pGWB1 (Nakagawa et al., 2007), in which *PHT1.2* had been subcloned (Kirchsteiger et al., 2012), using primers described in Supplemental Table S9 to introduce *Hin*dIII and *Spe*I sites, giving *PHT:GAPCp1*. For construct *HS:GAPCp1*, the *35S* promoter of *35S:GAPCp1* was exchanged for an 852-bp fragment corresponding to the promoter region of gene *HSP18.2*, giving the vector *HS:GAPCp1*. The *HSP18.2* promoter was obtained by digestion of the PTT101 plasmid (Matsuhara et al., 2000) with enzymes *Spe*I and *Hin*dIII. For *GUS-GFP* gene promoter-reporter fusions, a 1.5-kb fragment corresponding to the native *GAPCp1* promoter (−1,521 to +18 relative to the *GAPCp1* translation start) was placed before the 5′-*GUS-GFP*-3′ gene fusion in pCAMBIA1303 as described previously (Muñoz-Bertomeu et al., 2009). All PCR-derived constructs were verified by DNA sequencing.

Arabidopsis plants were transformed with the different constructs by the floral dipping method (Clough and Bent, 1998) with *Agrobacterium tumefaciens* carrying *pSOUP*. As *gapcp1gapcp2* is sterile, the progeny of heterozygous plants (heterozygous for *GAPCp1* and homozygous for the mutant allele of *GAPCp2*) were transformed with the different constructs. Transformants were selected by antibiotic selection, while homozygous *gapcp1gapcp2*, heterozygous (homozygous for *GAPCp1* and heterzygous for *GAPCp2*), and the wild type were identified by PCR genotyping using gene-specific primers (RP and LP) and left border primers of the T-DNA insertions described in Supplemental Table S9. Several independent single-insertion homozygous T3 lines were obtained for all the different constructs. After characterization by qRT-PCR, two to three different lines were selected for further analyses depending on the experiment. Syngenic wild-type lines were used as controls for our studies.

qRT-PCR and Microarrays

qRT-PCR was performed as described previously (Cascales-Miñana et al., 2013). Each reaction was performed in triplicate. Data are means of three biological samples. PCR amplification specificity was confirmed with a heat dissociation curve (from 60°C to 95°C). The efficiency of the PCR was calculated, and different internal standards were selected (Czechowski et al., 2005) depending on the primers' efficiency. Relative mRNA abundance was calculated using the comparative cycle threshold method according to Pfaffl (2001). Primers used for PCRs are listed in Supplemental Table S9.

For microarray experiments, total RNAs from three pools of 21-d-old roots and AP (*gapcp1gapcp2* and the wild type or *gapcp1gapcp2* and *gapcp1gapcp2 HS:GAPCp1*), vertically grown on one-fifth-strength MS plates as described above, were extracted using the total RNA isolation kit (Macherey-Nagel). For the induction experiments, *gapcp1gapcp2* and *gapcp1gapcp2 HS:GAPCp1* were treated for 1 h at 37°C 4 h before sampling. RNA integrity was determined using RNA 6000 Nano Labchips in an Agilent 2100 Bioanalyzer following the manufacturer's protocol. RNA amplification, labeling, and hybridization were done as described by Bueso et al. (2007). The Arabidopsis oligonucleotide microarrays were obtained from David Galbraith (http://www.ag.arizona.edu/microarray/). The chosen design for the microarray experiments was a balanced block design, with three to four biological replicates and two dye swaps. The microarray data analysis was done as described by Bissoli et al. (2012). Genes were selected as differentially expressed when expression was at least 1.5-fold higher or lower than the controls and the false discovery rate was lower than 10% when applying the significance analysis. Significant functional categories were selected using the Munich Information Center for Protein Sequences software (http://mips.helmholtz-muenchen.de/proj/funcatDB/). Microarray data were submitted to the ArrayExpress database (http://www.ebi.ac.uk/arrayexpress) with the accession number E-MTAB-3262 and are listed in Supplemental Table S9.

Enzyme Assays, Metabolite Determination, and Ion Analysis

APs and roots of 18- to 21-d-old wild-type and different *gapcp1gapcp2* transgenic lines were used for the determination of metabolite content. Starch

and total soluble sugars were determined with the ENZYTEC starch kit (R-Biopharm). Protein content was quantified using the Bio-Rad protein assay kit. The levels of other metabolites were determined in derivatized methanol extracts by gas chromatography-mass spectrometry following the protocol defined by Lisec et al. (2006). Macronutrients and micronutrients were determined according to Quiñones et al. (2011). Ammonium content was determined according to Saraiketa et al. (2014). Phosphoglycerate kinase activity was determined according to Brandina et al. (2006).

Western Blots

Crude protein extracts from whole wild-type and different *gapcp1gapcp2* transgenic lines were obtained by harvesting 0.5 g of plant material in liquid nitrogen and grinding in 1 mL of ice-cold homogenization buffer as described previously (Cascales-Miñana et al., 2013). Proteins were electrotransferred onto Immune-Blot nitrocellulose membranes (Bio-Rad) using the Mini Tran-Blot Cell (Bio-Rad) for 1 h at 100 V with the transfer buffer (17 mM Tris, 192 mM Gly, and 20% [v/v] methanol). Immunoblots were probed with anti-GFP antibodies (Molecular Probes; A-11122) or a specific antibody raised against a 16-amino acid specific epitope (Ac-KGSINVIDDSTLEINC-amide) of GAPCp1. Ponceau S-stained membranes or nonspecific bands are shown as loading controls. Cross-reacting bands were detected using the ECL Select Western-Blotting Detection Reagent Kit (Amersham Biosciences).

Microscopy

For GUS activity assays, roots were processed as described (Muñoz-Bertomeu et al., 2009). Images were acquired with a Leica DM1000 microscope and a Leica DC350 digital camera. GFP fluorescence was observed with a confocal microscope (Leica TCS-SP).

Statistics

Experimental values represent means and SE, and *n* represents the number of independent samples. *P* values were calculated with Student's *t* test (two tailed) using Microsoft Excel. The level of significance was fixed at 5% (0.05). PCA of metabolomic data was performed using the Excel add-in Multibase package (Numerical Dynamics). For PCA, relative values normalized to the mean response calculated for the wild type were scaled by SD prior to the analysis. The first principal component was used for the ANOVA of different lines, with Tukey's posthoc tests to differentiate between the lines.

Sequence data from this article can be found in the Arabidopsis Genome Initiative data libraries under accession numbers At1g79530 (*GAPCp1*) and At1g16300 (*GAPCp2*).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Molecular and physiological analysis of GAPCp isoforms.

Supplemental Figure S2. Cellular expression of GAPCp1 under the control of the *RBCS* and the *35S* promoters.

Supplemental Figure S3. Quantification of changes in transcript level using qRT-PCR.

Supplemental Figure S4. Molecular and phenotypical analyses of *gapcp1-gapcp2* expressing GAPCp1-GFP under the control of the *HS* promoter.

Supplemental Table S1. Metabolite levels in the aerial parts of wild-type, *gapcp1gapcp2* (*g1g2*), *g1g2 RBCS:GAPCp1*, and *g1g2 35S:GAPCp1* plants sampled at the end of the light period.

Supplemental Table S2. Metabolite levels in the aerial parts of wild-type, *gapcp1gapcp2* (*g1g2*), and *g1g2 RBCS:GAPCp1* plants grown in standard medium or supplemented with either 2% Suc or 0.1 mM Ser.

Supplemental Table S3. Metabolite levels in aerial parts and roots of wild-type, *gapcp1gapcp2* (*g1g2*), *g1g2 RBCS:GAPCp1*, and *g1g2 PHT:GAPCp1* sampled at the middle of the light period.

Supplemental Table S4. Metabolite levels in aerial parts and roots of wild-type and *gapcp1gapcp2 HS:GAPCp1* plants.

Supplemental Table S5. List of genes mentioned in this work whose expression was misregulated in *gapcp1gapcp2* (*g1g2*) or *g1g2 HS:GAPCp1* following microarray experiments.

Supplemental Table S6. Macronutrient and micronutrient concentrations in aerial parts and roots of wild-type, *gapcp1gapcp2* (*g1g2*), and *g1g2 PHT:GAPCp1* plants.

Supplemental Table S7. Effect of 1-h treatment at 37°C on metabolite levels in aerial parts and roots of *gapcp1gapcp2* plants.

Supplemental Table S8. Functional categories of genes differentially expressed in the aerial parts and roots of *gapcp1gapcp2 HS:GAPCp1* after GAPCp1 induction as compared with *gapcp1gapcp2*.

Supplemental Table S9. List of primers used in this work.

Supplemental Materials S1. Genes differentially expressed in transgenic and mutant lines.

LITERATURE CITED

Andre C, Benning C (2007) Arabidopsis seedlings deficient in a plastidic pyruvate kinase are unable to utilize seed storage compounds for germination and establishment. Plant Physiol **145:** 1670–1680

Andre C, Froehlich JE, Moll MR, Benning C (2007) A heteromeric plastidic pyruvate kinase complex involved in seed oil biosynthesis in *Arabidopsis*. Plant Cell **19:** 2006–2022

Andriotis VM, Kruger NJ, Pike MJ, Smith AM (2010) Plastidial glycolysis in developing Arabidopsis embryos. New Phytol **185:** 649–662

Backhausen JE, Vetter S, Baalmann E, Kitzmann C, Scheibe R (1998) NAD-dependent malate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase isoenzymes play an important role in dark metabolism of various plastid types. Planta **205:** 359–366

Baek D, Jin Y, Jeong JC, Lee HJ, Moon H, Lee J, Shin D, Kang CH, Kim DH, Nam J, et al (2008) Suppression of reactive oxygen species by glyceraldehyde-3-phosphate dehydrogenase. Phytochemistry **69:** 333–338

Baud S, Wuillème S, Dubreucq B, de Almeida A, Vuagnat C, Lepiniec L, Miquel M, Rochat C (2007) Function of plastidial pyruvate kinases in seeds of *Arabidopsis thaliana*. Plant J **52:** 405–419

Benstein RM, Ludewig K, Wulfert S, Wittek S, Gigolashvili T, Frerigmann H, Gierth M, Flügge UI, Krueger S (2013) *Arabidopsis* phosphoglycerate dehydrogenase1 of the phosphoserine pathway is essential for development and required for ammonium assimilation and tryptophan biosynthesis. Plant Cell **25:** 5011–5029

Bissoli G, Niñoles R, Fresquet S, Palombieri S, Bueso E, Rubio L, García-Sánchez MJ, Fernández JA, Mulet JM, Serrano R (2012) Peptidyl-prolyl *cis-trans* isomerase ROF2 modulates intracellular pH homeostasis in Arabidopsis. Plant J **70:** 704–716

Brandina I, Graham J, Lemaitre-Guillier C, Entelis N, Krasheninnikov I, Sweetlove L, Tarassov I, Martin RP (2006) Enolase takes part in a macromolecular complex associated to mitochondria in yeast. Biochim Biophys Acta **1757:** 1217–1228

Bueso E, Alejandro S, Carbonell P, Perez-Amador MA, Fayos J, Bellés JM, Rodriguez PL, Serrano R (2007) The lithium tolerance of the Arabidopsis *cat2* mutant reveals a cross-talk between oxidative stress and ethylene. Plant J **52:** 1052–1065

Cascales-Miñana B, Muñoz-Bertomeu J, Flores-Tornero M, Anoman AD, Pertusa J, Alaiz M, Osorio S, Fernie AR, Segura J, Ros R (2013) The phosphorylated pathway of serine biosynthesis is essential both for male gametophyte and embryo development and for root growth in *Arabidopsis*. Plant Cell **25:** 2084–2101

Chellamuthu VR, Ermilova E, Lapina T, Lüddecke J, Minaeva E, Herrmann C, Hartmann MD, Forchhammer K (2014) A widespread glutamine-sensing mechanism in the plant kingdom. Cell **159:** 1188–1199

Chen M, Thelen JJ (2010) The plastid isoform of triose phosphate isomerase is required for the postgerminative transition from heterotrophic to autotrophic growth in *Arabidopsis*. Plant Cell **22:** 77–90

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J **16:** 735–743

Curtis MD, Grossniklaus U (2003) A Gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol **133:** 462–469

Czechowski T, Stitt M, Altmann T, Udvardi MK, Scheible WR (2005) Genome-wide identification and testing of superior reference genes for transcript normalization in Arabidopsis. Plant Physiol **139:** 5–17

Fait A, Nesi AN, Angelovici R, Lehmann M, Pham PA, Song L, Haslam RP, Napier JA, Galili G, Fernie AR (2011) Targeted enhancement of glutamate-to-γ-aminobutyrate conversion in Arabidopsis seeds affects carbon-nitrogen balance and storage reserves in a development-dependent manner. Plant Physiol **157:** 1026–1042

Guo L, Devaiah SP, Narasimhan R, Pan X, Zhang Y, Zhang W, Wang X (2012) Cytosolic glyceraldehyde-3-phosphate dehydrogenases interact with phospholipase Dδ to transduce hydrogen peroxide signals in the *Arabidopsis* response to stress. Plant Cell **24:** 2200–2212

Hajirezaei MR, Biemelt S, Peisker M, Lytovchenko A, Fernie AR, Sonnewald U (2006) The influence of cytosolic phosphorylating glyceraldehyde 3-phosphate dehydrogenase (GAPC) on potato tuber metabolism. J Exp Bot **57:** 2363–2377

Han S, Wang Y, Zheng X, Jia Q, Zhao J, Bai F, Hong Y, Liu Y (2015) Cytoplastic glyceraldehyde-3-phosphate dehydrogenases interact with ATG3 to negatively regulate autophagy and immunity in *Nicotiana benthamiana*. Plant Cell **27:** 1316–1331

Kaplan F, Kopka J, Sung DY, Zhao W, Popp M, Porat R, Guy CL (2007) Transcript and metabolite profiling during cold acclimation of Arabidopsis reveals an intricate relationship of cold-regulated gene expression with modifications in metabolite content. Plant J **50:** 967–981

Kim JW, Dang CV (2005) Multifaceted roles of glycolytic enzymes. Trends Biochem Sci **30:** 142–150

Kim JY, Rim Y, Wang J, Jackson D (2005) A novel cell-to-cell trafficking assay indicates that the KNOX homeodomain is necessary and sufficient for intercellular protein and mRNA trafficking. Genes Dev **19:** 788–793

Kirchsteiger K, Ferrández J, Pascual MB, González M, Cejudo FJ (2012) NADPH thioredoxin reductase C is localized in plastids of photosynthetic and nonphotosynthetic tissues and is involved in lateral root formation in *Arabidopsis*. Plant Cell **24:** 1534–1548

Kleczkowski LA, Randall DD (1986) Thiol-dependent regulation of glycerate metabolism in leaf extracts: the role of glycerate kinase in C_4 plants. Plant Physiol **81:** 656–662

Li B, Li G, Kronzucker HJ, Baluška F, Shi W (2014) Ammonium stress in Arabidopsis: signaling, genetic loci, and physiological targets. Trends Plant Sci **19:** 107–114

Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR (2006) Gas chromatography mass spectrometry-based metabolite profiling in plants. Nat Protoc **1:** 387–396

Liu TY, Aung K, Tseng CY, Chang TY, Chen YS, Chiou TJ (2011) Vacuolar Ca^{2+}/H^+ transport activity is required for systemic phosphate homeostasis involving shoot-to-root signaling in Arabidopsis. Plant Physiol **156:** 1176–1189

Matsuhara S, Jingu F, Takahashi T, Komeda Y (2000) Heat-shock tagging: a simple method for expression and isolation of plant genome DNA flanked by T-DNA insertions. Plant J **22:** 79–86

May A, Berger S, Hertel T, Köck M (2011) The *Arabidopsis thaliana* phosphate starvation responsive gene *AtPPsPase1* encodes a novel type of inorganic pyrophosphatase. Biochim Biophys Acta **1810:** 178–185

Mudge SR, Rae AL, Diatloff E, Smith FW (2002) Expression analysis suggests novel roles for members of the Pht1 family of phosphate transporters in *Arabidopsis*. Plant J **31:** 341–353

Muñoz-Bertomeu J, Cascales-Miñana B, Irles-Segura A, Mateu I, Nunes-Nesi A, Fernie AR, Segura J, Ros R (2010) The plastidial glyceraldehyde-3-phosphate dehydrogenase is critical for viable pollen development in Arabidopsis. Plant Physiol **152:** 1830–1841

Muñoz-Bertomeu J, Cascales-Miñana B, Mulet JM, Baroja-Fernández E, Pozueta-Romero J, Kuhn JM, Segura J, Ros R (2009) Plastidial glyceraldehyde-3-phosphate dehydrogenase deficiency leads to altered root development and affects the sugar and amino acid balance in Arabidopsis. Plant Physiol **151:** 541–558

Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K, Matsuoka K, Jinbo T, Kimura T (2007) Development of

series of Gateway binary vectors, pGWBs, for realizing efficient construction of fusion genes for plant transformation. J Biosci Bioeng **104:** 34–41

Nishizawa A, Yabuta Y, Shigeoka S (2008) Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. Plant Physiol **147:** 1251–1263

Nussaume L, Kanno S, Javot H, Marin E, Pochon N, Ayadi A, Nakanishi TM, Thibaud MC (2011) Phosphate import in plants: focus on the PHT1 transporters. Front Plant Sci **2:** 83

Petersen J, Brinkmann H, Cerff R (2003) Origin, evolution, and metabolic role of a novel glycolytic GAPDH enzyme recruited by land plant plastids. J Mol Evol **57:** 16–26

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res **29:** e45

Pick TR, Bräutigam A, Schulz MA, Obata T, Fernie AR, Weber AP (2013) PLGG1, a plastidic glycolate glycerate transporter, is required for photorespiration and defines a unique class of metabolite transporters. Proc Natl Acad Sci USA **110:** 3185–3190

Plaxton WC (1996) The organization and regulation of plant glycolysis. Annu Rev Plant Physiol Plant Mol Biol **47:** 185–214

Prabhakar V, Löttgert T, Geimer S, Dörmann P, Krüger S, Vijayakumar V, Schreiber L, Göbel C, Feussner K, Feussner I, et al (2010) Phosphoenolpyruvate provision to plastids is essential for gametophyte and sporophyte development in *Arabidopsis thaliana*. Plant Cell **22:** 2594–2617

Quiñones A, Martínez-Alcántara B, San-Francisco S, García-Mina JM, Legaz F (2011) Methyl xanthine as a potential alternative to gibberellic acid in enhancing fruit set and quality in clementine citrus trees in Spain. Exp Agric **47:** 159–171

Rius SP, Casati P, Iglesias AA, Gomez-Casati DF (2008) Characterization of Arabidopsis lines deficient in GAPC-1, a cytosolic NAD-dependent glyceraldehyde-3-phosphate dehydrogenase. Plant Physiol **148:** 1655–1667

Ros R, Muñoz-Bertomeu J, Krueger S (2014) Serine in plants: biosynthesis, metabolism, and functions. Trends Plant Sci **19:** 564–569

Sarasketa A, González-Moro MB, González-Murua C, Marino D (2014) Exploring ammonium tolerance in a large panel of Arabidopsis thaliana natural accessions. J Exp Bot **65:** 6023–6033

Scholl RL, May ST, Ware DH (2000) Seed and molecular resources for Arabidopsis. Plant Physiol **124:** 1477–1480

Taji T, Ohsumi C, Iuchi S, Seki M, Kasuga M, Kobayashi M, Yamaguchi-Shinozaki K, Shinozaki K (2002) Important roles of drought- and cold-inducible genes for galactinol synthase in stress tolerance in *Arabidopsis thaliana*. Plant J **29:** 417–426

Tokunaga H, Kojima M, Kuroha T, Ishida T, Sugimoto K, Kiba T, Sakakibara H (2012) Arabidopsis lonely guy (LOG) multiple mutants reveal a central role of the LOG-dependent pathway in cytokinin activation. Plant J **69:** 355–365

Toujani W, Muñoz-Bertomeu J, Flores-Tornero M, Rosa-Téllez S, Anoman AD, Alseekh S, Fernie AR, Ros R (2013) Functional characterization of the plastidial 3-phosphoglycerate dehydrogenase family in Arabidopsis. Plant Physiol **163:** 1164–1178

Van der Straeten D, Rodrigues-Pousada RA, Goodman HM, Van Montagu M (1991) Plant enolase: gene structure, expression, and evolution. Plant Cell **3:** 719–735

Vescovi M, Zaffagnini M, Festa M, Trost P, Lo Schiavo F, Costa A (2013) Nuclear accumulation of cytosolic glyceraldehyde-3-phosphate dehydrogenase in cadmium-stressed Arabidopsis roots. Plant Physiol **162:** 333–346

Weber AP, Schwacke R, Flügge UI (2005) Solute transporters of the plastid envelope membrane. Annu Rev Plant Biol **56:** 133–164

Zaffagnini M, Fermani S, Costa A, Lemaire SD, Trost P (2013) Plant cytoplasmic GAPDH: redox post-translational modifications and moonlighting properties. Front Plant Sci **4:** 450

Zhang L, Tan Q, Lee R, Trethewy A, Lee YH, Tegeder M (2010) Altered xylem-phloem transfer of amino acids affects metabolism and leads to increased seed yield and oil content in *Arabidopsis*. Plant Cell **22:** 3603–3620