

RESEARCH PAPER

***Arabidopsis* plants deficient in plastidial glyceraldehyde-3-phosphate dehydrogenase show alterations in abscisic acid (ABA) signal transduction: interaction between ABA and primary metabolism**

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Abstract

Abscisic acid (ABA) controls plant development and regulates plant responses to environmental stresses. A role for ABA in sugar regulation of plant development has also been well documented although the molecular mechanisms connecting the hormone with sugar signal transduction pathways are not well understood. In this work it is shown that *Arabidopsis thaliana* mutants deficient in plastidial glycolytic glyceraldehyde-3-phosphate dehydrogenase (*gapcp1gapcp2*) are ABA insensitive in growth, stomatal closure, and germination assays. The ABA levels of *gapcp1gapcp2* were normal, suggesting that the ABA signal transduction pathway is impaired in the mutants. ABA modified *gapcp1gapcp2* gene expression, but the mutant response to the hormone differed from that observed in wild-type plants. The gene expression of the transcription factor ABI4, involved in both sugar and ABA signalling, was altered in *gapcp1gapcp2*, suggesting that their ABA insensitivity is mediated, at least partially, through this transcriptional regulator. Serine supplementation was able partly to restore the ABA sensitivity of *gapcp1gapcp2*, indicating that amino acid homeostasis and/or serine metabolism may also be important determinants in the connections of ABA with primary metabolism. Overall, these studies provide new insights into the links between plant primary metabolism and ABA signalling, and demonstrate the importance of plastidial glycolytic glyceraldehyde-3-phosphate dehydrogenase in these interactions.

Key words: ABA, ABA signal transduction, *Arabidopsis*, GAPCp, glyceraldehyde-3-phosphate dehydrogenase, glycolysis, sugar signalling.

Introduction

The phytohormone abscisic acid (ABA) controls the development of plants and plays a crucial role in their survival when faced with adverse environmental conditions, such as salt and water stress (Schroeder *et al.*, 2001; Himmelbach *et al.*, 2003). The genetic dissection of ABA signalling has now identified central players of this signal transduction pathway including ABA receptors (Shen *et al.*, 2006; Ma *et al.*, 2009; Melcher *et al.*, 2009; Miyazono *et al.*, 2009; Pandey *et al.*, 2009; Park *et al.*, 2009; Santiago *et al.*, 2009; Cutler *et al.*, 2010; Nishimura *et al.*, 2010), the group A

Abbreviations: ABA, abscisic acid; *gapcp1 gapcp2*, *Arabidopsis* plastidial glyceraldehyde-3-phosphate dehydrogenase double mutant; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GAPC, cytosolic glycolytic glyceraldehyde-3-phosphate dehydrogenase; *G1g1.1 g2.1g2.1*, population of seeds from heterozygous plants (*GAPCp1gapcp1.1 gapcp2.1gapcp2.1*); GAPCp, plastidial glycolytic glyceraldehyde-3-phosphate dehydrogenase; MS, Murashige and Skoog; Oex-GAPCp, plants overexpressing GAPCp; WT, wild type.

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protein phosphatases type 2C, which negatively regulate ABA signalling (Meyer *et al.*, 1994; Leung *et al.*, 1997), and the SNF1-related kinase 2 (SnRK2 kinases) which are positive regulators (Yoshida *et al.*, 2006; Vlad *et al.*, 2009) of downstream substrates such as AREB/ABF transcription factors (Furihata *et al.*, 2006).

A role for ABA in sugar regulation of plant development has been well documented (Arenas-Huertero *et al.*, 2000; Leon and Sheen, 2003; Rolland *et al.*, 2006; Rook *et al.*, 2006). Some of the *Arabidopsis* sugar signalling mutants are allelic to ABA biosynthetic or signalling mutants, and many ABA biosynthetic mutants also have sugar phenotypes (Arenas-Huertero *et al.*, 2000; Rolland *et al.*, 2006; Ramon *et al.*, 2008). The two main substrates for glycolysis in plants, sucrose and starch (Plaxton, 1996), are subjected to large diurnal changes in their concentrations. Thus, sugar levels and glycolysis need to be highly coordinated to provide metabolic flexibility that can respond to the differential demands of plant development and to environmental stress acclimation. In spite of considerable evidence corroborating the interactions between sugars and ABA, information about the role played by glycolysis in these interactions is lacking.

The most well known function of glycolysis is to provide energy and precursors for anabolic processes (Plaxton, 1996). In recent years, additional non-glycolytic functions, such as the regulation of transcription or apoptosis, have also been attributed to glycolytic enzymes in mammals and yeast (Kim and Dang, 2005). These additional functions of glycolytic enzymes suggest that links between metabolic sensors and development could be established directly through the enzymes that participate in metabolism (Kim and Dang, 2005). In this sense, a role for the plant glycolytic hexokinase as a glucose sensor has been reported (Moore *et al.*, 2003; Ramon *et al.*, 2008). On the other hand, the participation of enolase and aldolase in the plant response to salt tolerance (Barkla *et al.*, 2009) postulates a more direct function for glycolytic enzymes in acclimation to environmental stresses.

The glycolytic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reversibly converts glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate by coupling with the reduction of NAD⁺ to NADH. In mammals, GAPDHs have 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). A family of four genes encoding putative phosphorylating glycolytic GAPDHs (*GAPC1*, *GAPC2*, *GAPCp1*, and *GAPCp2*) is present in *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 the low gene expression level of GAPCps in *Arabidopsis*, *gapcp* double mutants (*gapcp1gapcp2*) show profound alterations of plant development, mainly arrested root development and sterility (Muñoz-Bertomeu *et al.*, 2009, 2010a, b). The strategic situation of GAPCp in the glycolytic pathway could make

this enzyme an important player not only for glycolysis but also for the generation of metabolites for other anabolic pathways (Plaxton, 1996; Ho and Saito, 2001; Andre *et al.*, 2007; Baud *et al.*, 2007). In this same vein, it was previously demonstrated that GAPCp deficiency affected the sugar and amino acid balance in *Arabidopsis* (Muñoz-Bertomeu *et al.*, 2009). Another pathway that could be affected by GAPCp deficiency is ABA biosynthesis. The substrate of GAPCp, glyceraldehyde-3-phosphate, is the first precursor of the methyl-erythritol phosphate pathway responsible for ABA biosynthesis in the plastids. Here evidence is provided that a deficiency in GAPCp is able to provide insensitivity to ABA. It is demonstrated that this insensitivity is not due to a modification of the ABA content but to an impaired ABA signal transduction in the GAPCp-deficient mutant. It is shown that the *gapcp1gapcp2* ABA insensitivity is caused by the sugar/amino acid imbalance displayed by the mutant. The ABA insensitivity of *gapcp1gapcp2* could be mediated, at least in part, via the transcriptional regulator ABI4. A specific role for amino acid homeostasis and/or serine metabolism in the ABA–sugar interaction is also discussed. This work provides new evidence for the links between plant primary metabolism and ABA signal transduction, and demonstrates the importance of GAPCps in these interactions.

Materials and methods

Plant material and growth conditions

Several *A. thaliana* (ecotype Columbia 0) *gapcp1* and *gapcp2* single and double T-DNA mutants were previously isolated as described in Muñoz-Bertomeu *et al.* (2009). For this work, mutant allele *gapcp1.1* (SAIL_390_G10) of the *At1g79530* gene, and *gapcp2.1* (SALK_137288) and *gapcp2.3* (SALK_037936) alleles of the *At1g16300* gene were used. Wild-type (WT) plants expressing a *GAPCp1* cDNA under the control of the 35S promoter (*Oex-GAPCp*) were previously obtained as described in Muñoz-Bertomeu *et al.* (2009).

Unless otherwise stated, seeds were sterilized and sown on 0.8% agar plates containing 1/5 Murashige and Skoog (MS) with Gamborg vitamins, and MES (0.9 g l⁻¹) 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, Japan) at 22 °C for a 16/8 h day/night photoperiod, 100 μmol m⁻² s⁻¹. Plates were supplemented with different concentrations of ABA and serine as indicated in the figure legends. Drought stress was imposed by placing the plates under the hood for 4 h before collecting the plants. For dormancy experiments, freshly harvested seeds were sown on plates containing 0.8% agar and 0.9 g l⁻¹ MES adjusted to pH 5.7 with TRIS. Because *gapcp1gapcp2* is sterile, a population of seeds from heterozygous plants (*GAPCp1gapcp1.1 gapcp2.1gapcp2.1*) was used for the germination experiments. This population, named *G1g1.1 g2.1g2.1*, contained 25% of double mutants (*gapcp1.1gapcp1.1 gapcp2.1gapcp2.1*), 50% of heterozygous plants (*GAPCp1-gapcp1.1 gapcp2.1gapcp2.1*), and 25% of single mutant plants (*GAPCp1GAPCp1 gapcp2.1gapcp2.1*). To score seed germination, the percentage of seeds that had germinated and developed fully green expanded cotyledons was determined. For the rest of the physiological and molecular studies, double homozygous *gapcp1-gapcp2* plants were identified in segregating populations of vertically grown plants by the root phenotype after 7–10 d as described (Muñoz-Bertomeu *et al.*, 2009). Then they were

subcultured on media containing different concentrations of ABA or serine for an additional 8–13 d as indicated in the figure legends and the tables. For stomata bioassay, some plantlets were grown in a Conviron growth chamber in pots filled with a (1:1, v/v) mixture of vermiculite and fertilized peat (KEKILA 50/50; kekkilä Iberia, S.L.) irrigated with demineralized water as needed.

Stomata bioassays

Stomatal responses were analysed in 3- to 4-week-old plants. Leaves were floated for 2.5 h in stomatal opening solution containing 50 mM KCl, 7 mM iminodiacetic acid, and 10 mM MES (pH 6.2). After incubation in 1 μ M and 10 μ M ABA for 2 h, leaf epidermal peels were obtained and the stomatal aperture was measured as described (Pei *et al.*, 1997). Control experiments were performed in parallel with no ABA added. *t*-Test (one-tailed, homoscedastic) *P*-values were calculated.

Microarrays

Fifteen day-old seedlings (*gapcp1gapcp2* and WT) vertically grown in 1/5 MS plates were used for the microarray experiments. On day 8 seedlings were subcultured on media containing 0.75 μ M ABA as described above. Total RNAs from three pools of 15-day-old seedlings (*gapcp1gapcp2* and WT) vertically grown in 1/5 MS plates at the same time were extracted using the RNeasy plant mini kit (Qiagen). RNA integrity was determined using RNA 6000 Nano Labchips[®] in an Agilent 2100 Bioanalyzer following the manufacturer's protocol. The purified RNA (8 μ g) was used to generate first-strand cDNA in a reverse transcription reaction (One-Cycle Target Labeling and Control Reagents; Affymetrix). After second-strand synthesis, the double-stranded cDNAs were used to generate cRNA via an *in vitro* transcription reaction. The cRNA was labelled with biotin, and 20 μ g of the labelled cRNA was fragmented. The size distribution of the cRNAs and fragmented cRNAs was assessed using an Eppendorf Biophotometer and electrophoresis. Fragmented cRNA (15 μ g) was added to 300 μ l of hybridization solution, and 200 μ l of this mixture was used for hybridization on *Arabidopsis* ATH1 Genome Arrays for 16 h at 45 °C. The standard wash and double-stain protocols (EukGE-WS2v5-450) were applied using an Affymetrix GeneChip Fluidics Station 450. The arrays (three replicates of each line) were scanned on an Affymetrix GeneChip scanner 3000. Fluorescence images were normalized with the software GCOS from Affymetrix. Expression data of ABA-treated seedlings were compared with those obtained from the non-ABA-treated controls grown under

the same conditions and at the same time (Muñoz-Bertomeu *et al.*, 2009). Data were analysed and compared using the dChip software (Li, 2008; <http://www.hsph.harvard.edu/~cli/complab/dchip/>) using a threshold criterion of 2-fold; *P* < 0.05. All generated data were uploaded to Gene Expression Omnibus (accession no. GSE21765) at NCBI.

Q-RT-PCR

Total RNA was extracted from seedlings as for microarray experiments. RNA was treated with RNase-free DNase (Promega). Then, 0.5 μ g of RNA was reverse transcribed using poly(T) primers and the first-strand cDNA synthesis Kit for RT-PCR (Roche) according to the manufacturer's instructions. Real-time quantitative PCR 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 μ l of the first-strand cDNA in a total volume of 25 μ l. Data are the mean of at least three biological samples. The specificity of the PCR amplification was confirmed with a heat dissociation curve (from 60 °C to 95 °C). Different internal standards were selected (Czechowski *et al.*, 2005) depending on the efficiency of the primers. Relative mRNA abundance was calculated using the comparative Ct method according to Pfaffl (2001). Primers used for quantitative RT-PCR (Q-RT-PCR) are listed in Supplementary Table S1 available at *JXB* online.

Metabolite determination

Starch and total soluble glucose were determined with the ENZYTEC starch kit (ATOM). ABA content was determined by liquid chromatography-electrospray tandem mass spectrometry (Durgbanshi *et al.*, 2005). Total glutathione was extracted and subsequently quantified by reverse-phase HPLC after derivatization with monobromobimane (Molecular Probes) following previously described methods (Dominguez-Solis *et al.*, 2001).

Results

gapcp1gapcp2 are ABA insensitivity

At the seedling stage, *gapcp1gapcp2* are smaller than WT plants when grown in a medium without ABA (Fig. 1A).

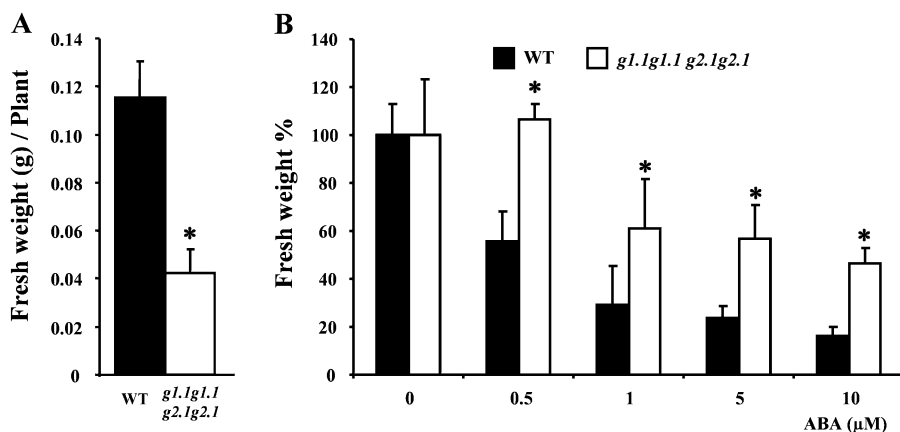


Fig. 1. The *gapcp1gapcp2* mutant shows ABA insensitivity to growth. (A) Fresh weight of the aerial part of 3-week-old *gapcp1gapcp2* seedlings (*g1.1g1.1 g2.1g2.1*) as compared with the wild type (WT). (B) Fresh weight (%) of the aerial part of 3-week-old *gapcp1gapcp2* seedlings (*g1.1g1.1 g2.1g2.1*) as compared with the WT under different ABA concentrations. Seedlings were treated for 13 d with the ABA concentrations indicated in the figure. Data are the mean \pm SD, *n* \geq 5 plates, each plate containing four (WT) and six (*g1.1g1.1 g2.1g2.1*) plants. *Significant at a *P*-value < 0.05 as compared with the WT. For simplicity, *g* stands for *gapcp*.

The fresh weight of 3-week-old *gapcp1gapcp2* plants grown in MS medium was almost three times lower than that of the WT (Fig. 1A). However, *gapcp1gapcp2* showed less sensitivity to growth inhibition by ABA. While WT growth was inhibited by ~50% by 0.5 μM ABA, the growth of *gapcp1gapcp2* was not significantly affected by this concentration (Fig. 1B). A 2-fold higher ABA concentration (1 μM) was necessary to achieve the same level of growth

inhibition in *gapcp1gapcp2* when compared with the WT (Fig. 1B).

It may be argued that *gapcp1gapcp2* ABA growth insensitivity could be associated with the slower growth rate of the mutant under non-ABA conditions as compared with the WT. To rule out this possibility other typical ABA responses were examined. Stomata bioassay experiments revealed that *gapcp1gapcp2* responds to ABA by closing stomata, but the response needs a higher ABA concentration if compared with the response of the WT (Fig. 2).

Seed germination assays were also performed. Since *gapcp1gapcp2* seeds are sterile, single *gapcp* mutants (*gapcp1* and *gapcp2*) and a mixed population of GAPCp-deficient seeds (single mutant, double mutant, and heterozygous), named *G1g1.1 g2.1g2.1*, originating from heterozygous mother plants (*GAPCp1gapcp1gapcp2gapcp2*), were used in these experiments. Seed dormancy was not altered by GAPCp deficiency as compared with the WT (Fig. 3A). However, germination of GAPCp-deficient seeds was insensitive to ABA. The ABA insensitivity phenotype was already observed in *gapcp* single mutants (Fig. 3B) and this phenotype was also observed in *G1g1.1 g2.1g2.1* (Fig. 3C). Plants overexpressing *GAPCp1* (*Oex-GAPCp1*) did not show hypersensitivity to ABA (Fig. 3C), which suggests that either only loss of GAPCp1 function affects the ABA response or that other compensatory and/or regulatory mechanisms are responsible for this lack of phenotype.

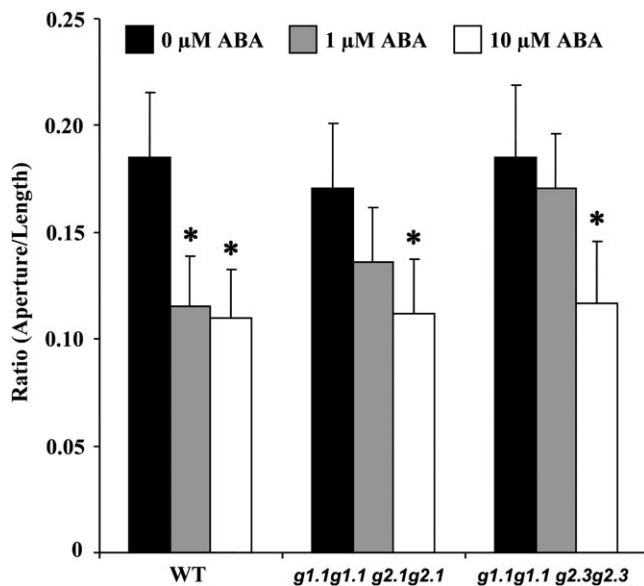


Fig. 2. The *gapcp1gapcp2* mutant shows ABA insensitivity to stomatal closing. ABA-induced stomatal closing in the wild type (WT) and in two different alleles of *gapcp1gapcp2* (*g1.1g1.1 g2.1g2.1*, *g1.1g1.1 g2.3g2.3*) plants. Data are the mean \pm SD, $n=30$ –40 stomata per experiment. *Significant at a P -value <0.05 as compared with non-ABA-treated plants. For simplicity, *g* stands for *gapcp*.

ABA and glutathione levels are not modified in gapcp1gapcp2

The ABA-insensitive phenotype of *gapcp1gapcp2* may indicate that this mutant is ABA deficient. The methylerythritol phosphate pathway responsible for ABA biosynthesis is located in the plastids. The first precursor in this

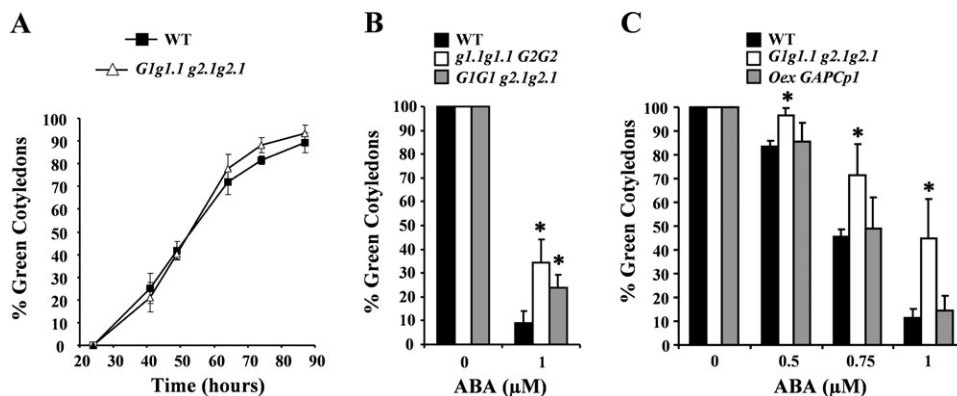


Fig. 3. The *gapcp1gapcp2* mutant shows ABA insensitivity during germination. (A) Germination of the wild type (WT) and a mixed population of GAPCp-deficient (double mutant, heterozygous and single mutant plants; *G1g1.1 g2.1g2.1*) seeds. Fresh seeds without a cold pre-treatment were plated in agar plates containing 0.2 g l^{-1} MES. (B) Germination of 10-day-old seeds from the WT and single mutants of *GAPCp1* (*g1.1g1.1 G2G2*) and *GAPCp2* (*G1G1 g2.1g2.1*) with and without 1 μM ABA. (C) Germination of 10-day-old WT seeds, a mixed population of GAPCp-deficient seeds (double mutant, heterozygous, and single mutant; *G1g1.1 g2.1g2.1*), and *GAPCp1*-overexpressing (*Oex-GAPCp1*) seeds under different ABA concentrations. Data are the mean \pm SD; each experiment consisted of at least four plates with 60 seeds each. Data from *Oex-GAPCp1* are from a representative line, but similar results were obtained with two other T_3 overexpressing lines. The experiment was repeated several times with different pools of seeds. *Significant at a P -value <0.05 as compared with WT seeds. For simplicity, *g* stands for *gapcp* and *G* for *GAPCp*.

pathway is glyceraldehyde-3-phosphate, the substrate of GAPCp. The ABA content in the aerial part of *gapcp1-gapcp2* did not differ significantly from that of the WT under non-drought stress conditions. In addition, the mutant was able to respond to imposed drought stress by increasing the ABA content similarly to the WT (Table 1).

gapcp1gapcp2 roots display serine deficiency (Muñoz-Bertomeu *et al.*, 2009). Since serine is the precursor of glycine and cysteine, both of which form part of the

glutathione molecule, the levels of this metabolite were also measured. The glutathione contents in both the aerial part and the roots of *gapcp1gapcp2* did not differ significantly from those of the WT (Table 1).

gapcp1gapcp2 plants display an altered gene expression in response to ABA

To assess the effect of ABA on the gene expression of *gapcp1gapcp2*, a microarray analysis comparing *gapcp1-gapcp2* and the WT after ABA treatment was performed. These data were compared with those obtained under identical conditions with non-ABA-treated plants (Muñoz-Bertomeu *et al.*, 2009). Full details of the microarray analysis are provided in Supplementary Table S2 at *JXB* online.

ABA treatment deregulated 321 genes (a >2-fold difference relative to the WT; $P < 0.05$) in *gapcp1gapcp2* as compared with the non-ABA-treated mutants and 193 genes in the WT as compared with the non-ABA-treated WT (Fig. 4A, and Supplementary Table S2 at *JXB* online). ABA-treated WT and *gapcp1gapcp2* only shared 24% of the deregulated genes. Thus, the gene expression response of *gapcp1gapcp2* to ABA differed from that of the WT, and could be related to the insensitivity phenotypes observed in the mutant.

Table 1. ABA (ng g⁻¹ DW) and glutathione (nmol g⁻¹ DW) content in the wild-type (WT) and *gapcp1gapcp2* (*g1.1g1.1 g2.1g2.1*) with or without drought stress

Three-week-old plants were grown in plates. For drought stress, plates were left open under the hood for 4 h before harvesting. Results are the mean \pm SD, $n \geq 3$.

Lines	ABA		Glutathione	
	Aerial part	Aerial part	Roots	Roots
WT (control)	26.6 \pm 1.4	2454 \pm 169	1586 \pm 57	
<i>g1.1g1.1 g2.1g2.1</i> (control)	24.1 \pm 1.7	2513 \pm 128	1627 \pm 81	
WT (drought stress)	179.8 \pm 5.3	–	–	
<i>g1.1g1.1 g2.1g2.1</i> (drought stress)	173.8 \pm 4.6	–	–	

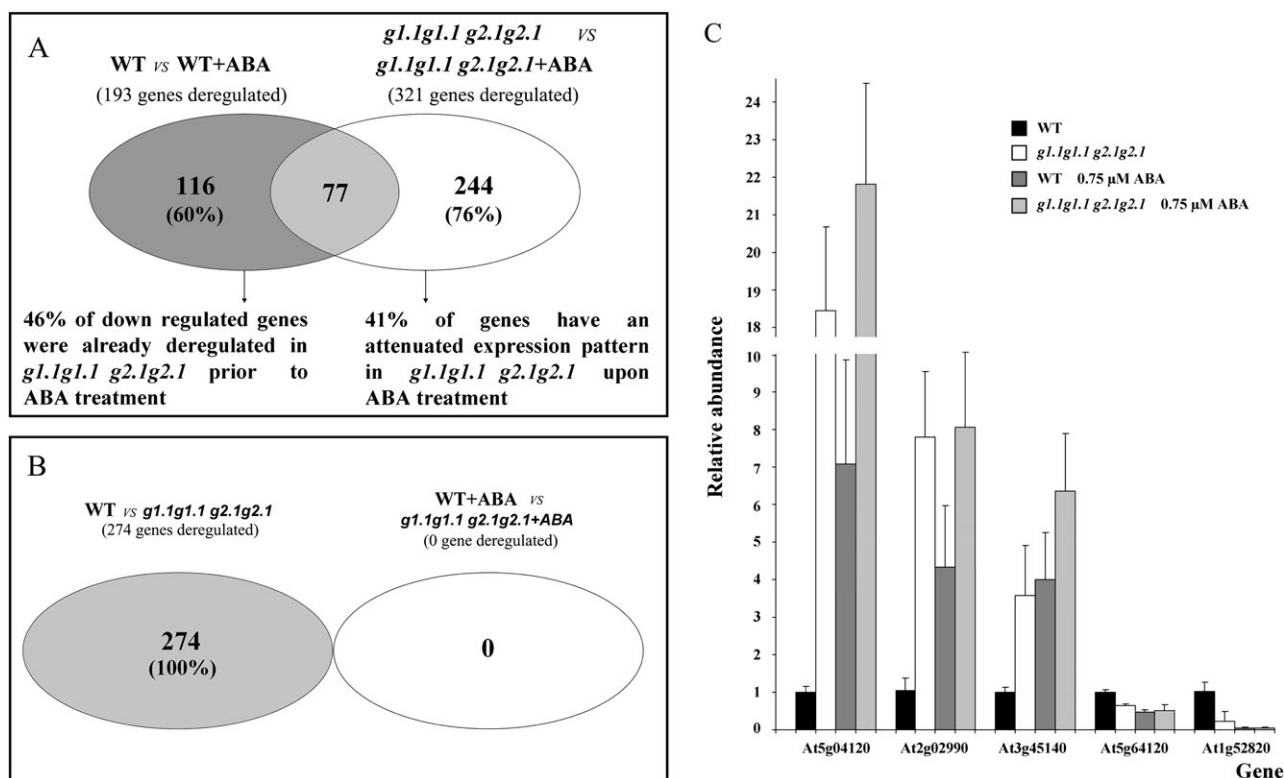


Fig. 4. ABA gene expression is deregulated in *gapcp1gapcp2*. (A) Comparison of the wild type (WT) versus WT+ABA and *gapcp1gapcp2* (*g1.1g1.1 g2.1g2.1*) versus *g1.1g1.1 g2.1g2.1*+ABA. (B) Comparison of the WT versus *g1.1g1.1 g2.1g2.1* and WT+ABA versus *g1.1g1.1 g2.1g2.1*+ABA. (C) Quantification of transcript level changes in *g1.1g1.1 g2.1g2.1* upon ABA treatment. RNA was extracted from 18-day-old *gapcp1gapcp2* and WT seedlings grown in the presence or absence of 0.75 μ M ABA for 10 d. The expression of a selection of up- and down-regulated genes in *gapcp1gapcp2* was quantified using real-time PCR. Data are the mean \pm SD; $n=3$. For simplicity, *g* stands for *gapcp*.

More than 270 genes were deregulated in *gapcp1gapcp2* as compared with the WT (Fig. 4B, and Supplementary Table S2). Surprisingly, after long-term ABA treatment there were no significant differences in gene expression between *gapcp1gapcp2* and the WT (Fig. 4B). Some of the genes deregulated by ABA in the WT, especially the down-regulated genes (46%), were already deregulated in *gapcp1gapcp2* prior to ABA treatment (Fig. 4A). Furthermore, in 41% of genes deregulated in *gapcp1gapcp2*, differences in gene expression between *gapcp1gapcp2* and the WT were attenuated by the hormone treatment (Fig. 4A). The effect of ABA on several genes deregulated in *gapcp1gapcp2* was reconfirmed by real-time Q-RT-PCR. As shown in Fig. 4C, the gene expression differences between *gapcp1gapcp2* and the WT were attenuated upon ABA treatment.

ABA affects carbohydrate metabolism and deregulates *ABI4* expression in *gapcp1gapcp2*

gapcp1gapcp2 present high levels of carbohydrates in both the aerial part and roots as compared with the WT (Muñoz-Bertomeu *et al.*, 2009). ABA treatment reduced both starch and total soluble glucose levels in the aerial part of *gapcp1gapcp2* (Fig. 5). The effect of ABA on the root carbohydrate level of *gapcp1gapcp2* was not as clear.

Although there was a trend to a reduction in the starch and an increase in the glucose content, these changes were not significant (Fig. 5). As the ABA-INSENSITIVE transcription factor *ABI4* has been described as a central transcriptional regulator connecting ABA with sugar signalling, the gene expression of *ABI4* was investigated in 48 h imbibed seeds ($\pm 0.25 \mu\text{M}$ ABA) from the WT and from a mixed population of GAPCp-deficient seeds (*G1g1.1 g2.1g2.1*). *ABI4* induction by ABA was reduced by GAPCp inactivation as compared with the WT (Fig. 6).

ABA insensitivity of *gapcp1gapcp2* is partially dependent on serine supply

It was previously demonstrated that serine supplementation to the growing medium restored normal carbohydrate levels in *gapcp1gapcp2* (Muñoz-Bertomeu *et al.*, 2009). In order to investigate whether the *gapcp1gapcp2* ABA insensitivity phenotype may be related to only its impaired carbohydrate metabolism, the ABA phenotypes of *gapcp1gapcp2* were investigated in those mutants supplemented with serine. The presence of serine in the growing medium abolished *gapcp1gapcp2* insensitivity to growth inhibition by ABA at all the concentrations used (Fig. 7). Serine also significantly reduced the ABA insensitivity of *gapcp1gapcp2* during germination (Fig. 8). In the presence of serine, the

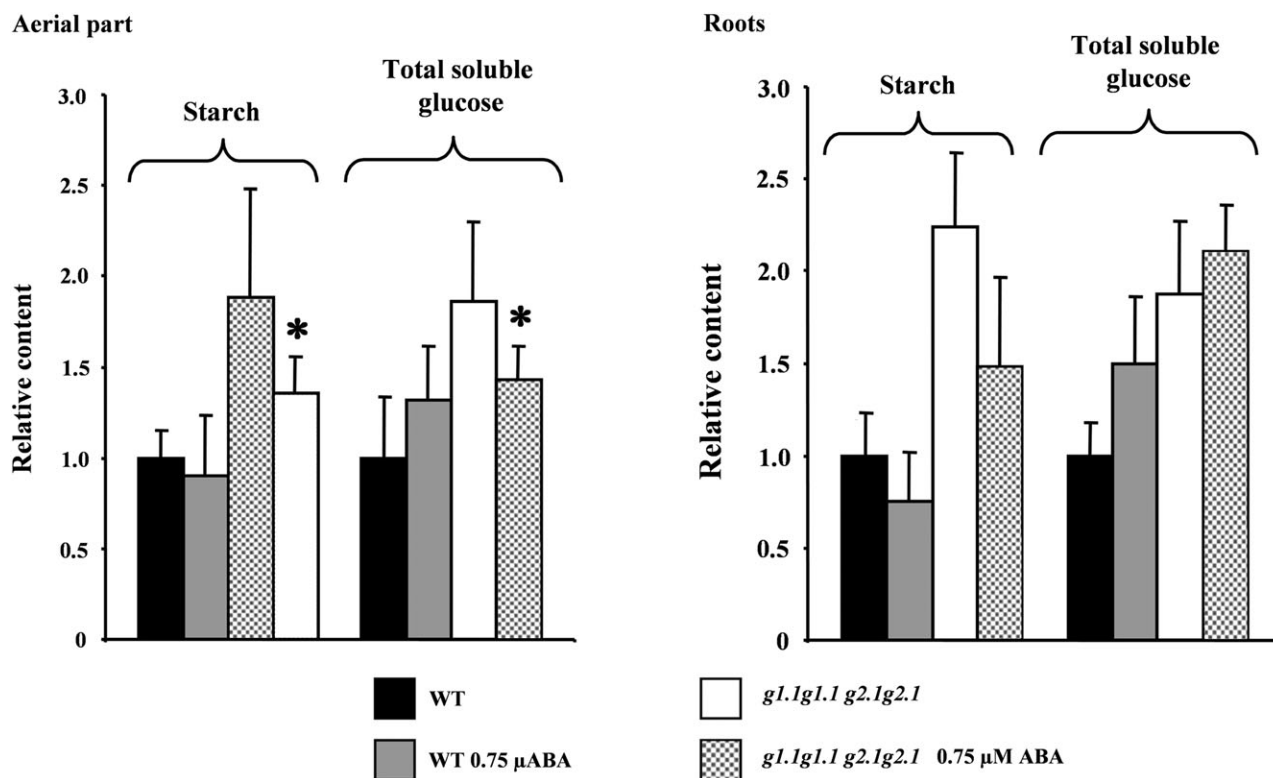


Fig. 5. ABA reduces sugar content in *gapcp1gapcp2*. Starch and total soluble glucose in the aerial part and roots of 3-week-old wild-type (WT) and *gapcp1gapcp2* (*g1.1g1.1 g2.1g2.1*) seedlings grown in the presence or absence of $0.75 \mu\text{M}$ ABA for 10 d. Values were normalized to the mean response of the WT in $\text{mg g fresh weight}^{-1}$ (starch, 1.75 ± 0.33 in the aerial part of the WT, 0.19 ± 0.05 in roots of the WT; soluble glucose, 0.31 ± 0.10 in the aerial part of the WT, 0.87 ± 0.15 in roots of the WT). Data are the mean \pm SD, $n \geq 3$; *Significant at a P -value < 0.05 as compared with non-ABA-treated plants. For simplicity, *g* stands for *gapcp*.

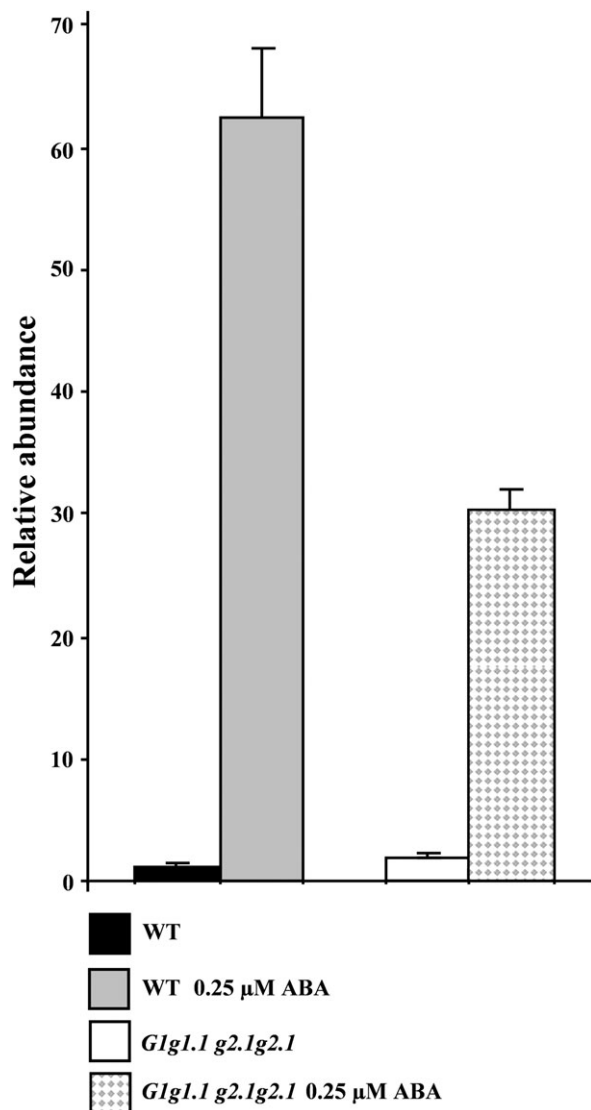


Fig. 6. *ABI4* expression is deregulated in *gapcp1gapcp2*. Quantification of changes in *ABI4* expression in seeds from the wild type (WT) and from a mixed population deficient in GAPCp (double mutant, heterozygous, and single mutant; *G1g1.1 g2.1g2.1*). RNA was extracted from seeds which had been imbibed for 48 h and germinated in the presence or absence of 0.25 μM ABA. *ABI4* expression was quantified using real-time PCR. Data are the mean ±SD; $n=3$. For simplicity, *g* stands for *gapcp* and *G* for *GAPCp*.

inhibition by ABA of *gapcp1gapcp2* germination did not differ from that of the WT not supplied with serine (Fig. 8). Interestingly, serine supplementation produced hypersensitivity to ABA in both the WT and *gapcp1gapcp2* (Fig. 8).

Discussion

gapcp1gapcp2 has an impaired ABA signal transduction pathway

gapcp1gapcp2 was insensitive to ABA, but the mutant's dormancy response was not altered as compared with the

WT, indicating that the ABA phenotype observed in the mutants is hormone specific. The impaired *gapcp1gapcp2* ABA response could indicate an alteration either of ABA biosynthesis or of the hormone signalling. The ABA levels in *gapcp1gapcp2* were normal, suggesting that GAPCp affects ABA signal transduction. In this sense, the gene expression response of *gapcp1gapcp2* to ABA differs from that of the WT. Thus, *GAPCp* inactivation does not eliminate gene responses to ABA but modifies them. The altered gene expression upon ABA treatment can be related to the ABA insensitivity of *gapcp1gapcp2* and suggests that ABA signal transduction in the mutant is impaired.

The ABA response of *gapcp1gapcp2* interferes with carbohydrate metabolism

The *gapcp1gapcp2* ABA insensitivity phenotype may relate to its impaired carbohydrate metabolism. Starch levels have been seen to decrease upon stress, such as cold (Nagao *et al.*, 2005), salt (Kempa *et al.*, 2007), or ABA treatment (Akihiro *et al.*, 2005). It could be argued that *gapcp1gapcp2* display more ABA insensitivity than the WT because they have a higher starch content than the WT under non-stress conditions. ABA would induce the mobilization of starch to obtain compatible solutes under stress conditions. In fact, the ABA treatment of *gapcp1gapcp2* was associated with a reduction in the starch and soluble glucose levels in the aerial part. This result indicates that *gapcp1gapcp2* respond to ABA. Furthermore, the results are a direct proof that ABA interferes with starch metabolism.

The transcription factor *ABI4* has been implicated in both ABA and sugar signal transduction cascades during early seedling development and could be one of the links between these two pathways (Acevedo-Hernandez *et al.*, 2005; Rook *et al.*, 2006; Bossi *et al.*, 2009). *ABI4* has also been implicated in the retrograde signalling between plastids and the nucleus (Koussevitzky, 2007). The induction of the *ABI4* gene by ABA in GAPCp-deficient seeds was attenuated as compared with that observed in the WT. These results indicate that the ABA insensitivity of *gapcp1gapcp2* may be mediated, at least at the initial developmental stages, through *ABI4*. Since these expression experiments were performed in a mixed population of single mutants, double mutants, and heterozygous seeds, much higher differences in *ABI4* expression should be expected between a homogeneous population of *gapcp1gapcp2* and WT seeds. Finally, the deregulation of *ABI4* expression in *gapcp1gapcp2* supports the microarray conclusions indicating that *GAPCp* inactivation modifies the ABA-induced gene response.

Involvement of serine metabolism in ABA signal transduction

As *ABI4* expression is narrowed down to the initial stages of seedling development, it is possible that other signalling molecules participate in the connection between sugar and ABA signal transduction. Serine supply restored normal

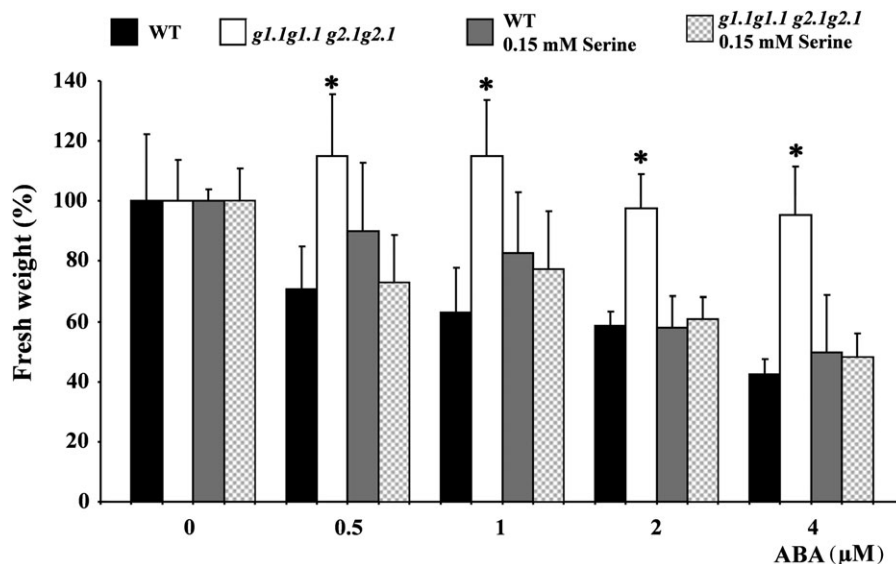


Fig. 7. *gapcp1gapcp2* ABA insensitivity to growth is abolished by serine supplementation. Fresh weight (%) of the aerial part of 18-day-old *gapcp1gapcp2* seedlings (*g1.1g1.1 g2.1g2.1*) as compared with the wild type (WT) under different concentrations of ABA and serine. Seedlings were treated for 9 d with the concentrations of ABA and serine indicated in the figure. Data are the mean \pm SD, $n \geq 5$ plates; each plate contained 4 (WT) and six (*g1.1g1.1 g2.1g2.1*) plants. *Significant at a P -value < 0.05 as compared with the WT. For simplicity, *g* stands for *gapcp*.

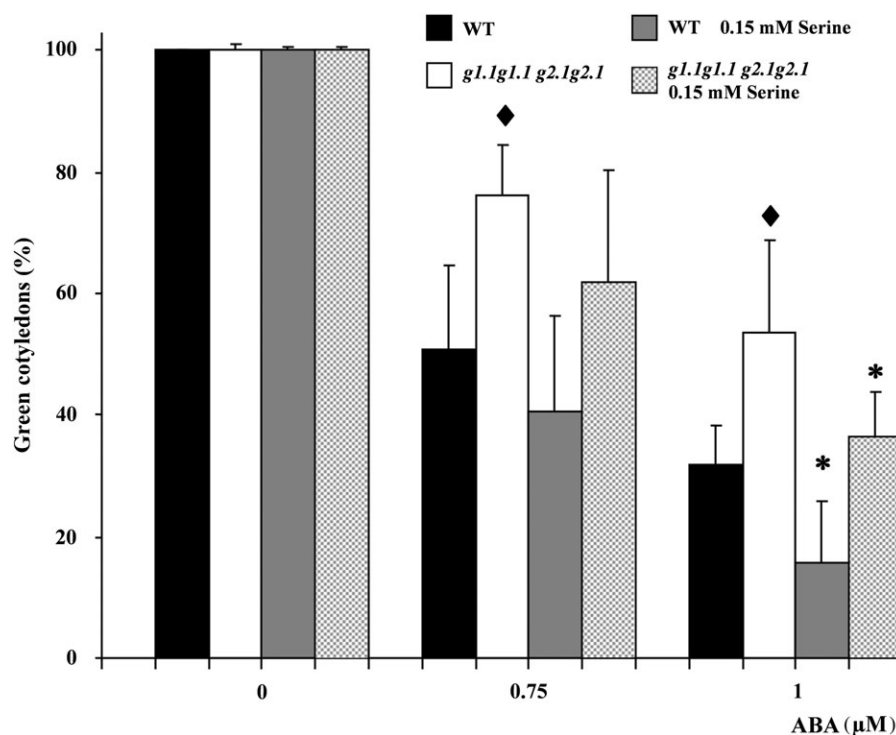


Fig. 8. Serine supplementation confers hypersensitivity to ABA during germination. Germination of 10-day-old seeds from wild-type (WT) and heterozygous *gapcp* (*G1g1.1 G2.1g2.1*) plants under different concentrations of ABA and serine. Data are the mean \pm SD; each experiment consisted of at least four plates with 60 seeds each. The experiment was repeated several times with different pools of seeds. ♦Significant at a P -value < 0.05 as compared with the non-serine-treated WT seeds. *Significant at a P -value < 0.05 as compared with the non-serine-treated seeds within each genotype. For simplicity, *g* stands for *gapcp* and *G* for *GAPCP*.

ABA growth sensitivity in *gapcp1gapcp2*, which could be due to its effect on carbohydrate metabolism, as already discussed. However, it could also be related, at least in part, to changes in the amino acid pools. A recent metabolomic

study has shown a correlation among drought stress, ABA production, and amino acid accumulation in *Arabidopsis* (Urano *et al.*, 2009). The total free amino acids in *gapcp1gapcp2* increased as compared with the control plants

(Muñoz-Bertomeu *et al.*, 2009). It is reasonable to think that an altered amino acid homeostasis provokes an impaired ABA transduction as a feedback.

In addition, a more specific effect of serine and/or derivatives on ABA signalling may also be considered. Serine was the only amino acid whose content was significantly lowered by 16% in the *gapcp1gapcp2* roots as compared with the WT (Muñoz-Bertomeu *et al.*, 2009). In this work, it was shown how serine supply increased the germination sensitivity of both the WT and *gapcp1gapcp2* to ABA, which suggests that this amino acid could be a link connecting ABA signal transduction and primary metabolism. Reactive oxygen species (ROS) participate in ABA signal transduction (Guan *et al.*, 2000; Pei *et al.*, 2000; Kwak *et al.*, 2003; Suhita *et al.*, 2004). As a result of serine deficiency in the mutant, the levels of glutathione, a central player in the scavenging of ROS, could be reduced. Thus, the ABA responses of *gapcp1gapcp2* could be affected by a compromised antioxidant capacity as compared with the WT. However, the glutathione levels were normal in both the aerial part and roots of *gapcp1gapcp2*, making this possibility improbable.

There is considerable evidence to suggest that serine may be involved in the responses of plants to various environmental stresses. For instance, an accumulation of serine in plants grown at low temperatures and elevated salinity has been reported (Ho and Saito, 2001, and references therein). In addition, the overexpression of the gene encoding 3-phosphoglycerate dehydrogenase, which catalyses the first step of the phosphorylated pathway of serine biosynthesis, improved the tolerance of *Arabidopsis* to salt and cold stresses, although the molecular mechanism involved remains unclear to date (Waditee *et al.*, 2007). It is suggested that the participation of serine in the responses of plants to salt and cold stresses could be mediated through the ABA signal transduction pathway.

In contrast to mutants, *Oex-GAPCp1* lines did not show an ABA phenotype. Although high levels of *GAPCp1* transcript are observed in *Oex-GAPCp1* lines (Supplementary Fig. S1 at *JXB* online), their carbohydrate and serine contents did not change significantly as compared with the WT (Muñoz-Bertomeu *et al.*, 2009). This could be due to a metabolic readjustment achieved either indirectly or directly through a specific regulation of GAPCp activity (post-transcriptional and/or post-translational regulation). Therefore, the lack of ABA phenotype of *Oex-GAPCp1* lines would support the conclusion that it is the alteration of the metabolic homeostasis observed in *gapcp1gapcp2* which impairs the ABA response.

Given the autotrophic nature of plants, the regulation of chloroplast/plastid functions is crucial for plant survival. The present results not only indicate that altered plastidial glycolysis impairs the signalling network of C, N, and ABA operating in *Arabidopsis* seedling development, but also provide evidence of the importance of GAPCps in these interactions. Evidence has also been provided for a link between the glycolytic pathway and the ABI4 transcription factor. Finally, the results suggest that serine (directly or

indirectly) may play a role in connecting primary metabolism and ABA signal transduction.

Accession numbers

Arabidopsis Genome Initiative locus identifiers of *Arabidopsis* genes used in this article are as follows: *GAPCp1* (At1g79530), *GAPCp2* (At1g16300).

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. Quantification of *GAPCp1* transcript level in the roots and aerial part of *GAPCp1*-overexpressing (*Oex-GAPCp1*) lines as compared with the wild type (WT). Data from a representative overexpressing line are presented.

Table S1. Primers used in this work

Table S2. Microarray data. The data were compared using the dChip software.

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