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The role of plastidic phosphoglucose isomerase in the response of *Arabidopsis thaliana* to volatile compounds emitted by pathogenic microorganisms

Tesis Doctoral para optar al grado de Doctor, presentada por:

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CERTIFICAN:

Que el trabajo titulado "The role of plastidic phosphoglucose isomerase in the response of *Arabidopsis thaliana* to volatile compounds emitted by pathogenic microorganisms" recogido en la presente memoria, ha sido realizado por Ángela Mª Sánchez López en el Instituto de Agrobiotecnología (UPNA/CSIC/Gobierno de Navarra) bajo su supervisión y que cumple las condiciones exigidas por la legislación vigente para optar al grado de Doctor. Ángela Mª Sánchez López ha disfrutado de una beca predoctoral FPI del Ministerio de Educación y Ciencia. Este trabajo ha sido financiado por los proyectos **BIO2010-182309 y BIO2013-49125-C2-1-P** de la Comisión Interministerial de Ciencia y Tecnología.

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RESUMEN

RESUMEN

El almidón es un homopolímero ramificado de residuos de glucosa unidos covalentemente a través de enlaces de tipo α-1,4 y α-1,6. Sintetizado en el plastidio, este polisacárido de reserva constituye la forma principal de almacenamiento de carbohidratos en plantas superiores y un determinante importante tanto del crecimiento de la planta como de su relación con el entorno. Está ampliamente aceptado que el proceso de biosíntesis del almidón en hojas tiene lugar exclusivamente en el cloroplasto. Según esta interpretación, el almidón es el producto final de una ruta metabólica conectada con el ciclo de Calvin-Benson (CBC) a través de la fosfoglucosa isomerasa plastidial (pPGI). Esta enzima cataliza la conversión de moléculas de fructosa-6-fosfato del CBC en moléculas de glucosa-6-fosfato, las cuales son metabolizadas en almidón a través de la acción combinada de la fosfoglucomutasa, la ADP-glucosa pirofosforilasa y la almidón sintasa.

Las plantas perciben estímulos bióticos mediante el reconocimiento de una gran cantidad de compuestos procedentes de los organismos con los que interactúan. En este sentido cabe destacar que los microorganismos de la rizosfera sintetizan una gran cantidad de sustancias que regulan el desarrollo y el crecimiento de la planta. Además, estos microorganismos emiten una amplia gama de compuestos volátiles (VCs) que actúan como "infoquímicos" en la comunicación entre la planta y el microorganismo. Estudios llevados a cabo por el grupo de investigación en el que he desarrollado este trabajo de tesis doctoral demostraron que los VCs emitidos por una amplia gama de microorganismos (incluyendo patógenos y especies que normalmente no interactúan con la planta) fomentan la acumulación de cantidades excepcionalmente elevadas almidón en la planta. Dada la falta de conocimiento sobre los mecanismos implicados en este fenómeno y teniendo en cuenta a su vez el papel importante que juega el almidón en la interacción de la planta con su entorno, en este trabajo de tesis doctoral investigué las bases moleculares implicadas en la respuesta de la planta a los VCs microbianos, prestando especial atención al papel que juega la pPGI en esta respuesta.

El **primer capítulo** de este trabajo describe la caracterización de dos mutantes (*pgi1-2* y *pgi1*-3) carentes de actividad pPGI. Ambos acumulan en sus hojas un 10-15% del almidón existente en hojas de plantas salvaje (WT). Contrariamente a lo que pudiera esperarse al tener en cuenta la interpretación clásica de la biosíntesis de almidón, análisis por microscopía revelaron la presencia de gránulos de almidón en

cloroplastos de las células del mesófilo de estos mutantes. Tanto *pgi1-2* como *pgi1-3* mostraron un crecimiento lento, una reducida capacidad fotosintética y un bajo balance NAD(P)H/NAD(P) con respecto a plantas WT. Estudios hormonómicos mostraron que el contenido de citoquininas (CKs) plastidiales en hojas *pgi1* es muy reducido con respecto al existente en hojas WT. Además la aplicación exógena de CKs revirtió el fenotipo de deficiencia de almidón de hojas de plantas *pgi1*. Los datos presentados en este trabajo indican que pPGI es un importante determinante de la fotosíntesis, el estado redox de la célula, el crecimiento y la acumulación de almidón en células del mesófilo como consecuencia de su implicación en la producción de intermediarios de la vía oxidativa de las pentosas fosfato/glicólisis necesarios para la síntesis CKs plastidiales y poder reductor.

El **capítulo 2** muestra que VCs emitidos por microorganismos filogenéticamente distantes (incluyendo bacterias y hongos beneficiosos y patógenos) promueven el crecimiento, la acumulación de niveles excepcionalmente elevados de almidón y la floración en varias especies de plantas, incluidos cultivos de interés agronómico. Además, plantas de Arabidopsis expuestas a VCs emitidos por el fitopatógeno *Alternaria alternata* experimentaron un incremento en la fotosíntesis y un aumento del contenido de CKs. La magnitud de este fenómeno fue reducida en el mutante 35S:AtCKX1 deficiente en CKs y en el mutante *ahk2/3* de señalización de CKs, proporcionando así evidencia de que este tipo de hormonas juega un papel importante en la respuesta de las plantas a VCs microbianos. El análisis transcriptómico de hojas de Arabidopsis expuestas a VCs de *A. alternata* reveló cambios en la expresión de genes regulados por luz y CKs implicados en la fotosíntesis, la floración, el crecimiento y el metabolismo del almidón. Sorprendentemente, una gran cantidad de genes diferencialmente expresados en plantas tratadas con VCs de *A. alternata* son genes cuya expresión se ve alterada también en plantas expuestas a VCs emitidos por la bacteria beneficiosa *Bacillus subtilis* GB03, sugiriendo que las plantas han desarrollado la capacidad de reaccionar a VCs emitidos por diferentes microorganismos a través de la activación o estimulación de mecanismos altamente conservados.

Para entender mejor los mecanismos implicados en las respuestas de las plantas a los VCs emitidos por microorganismos e investigar en qué medida pPGI está implicada en este fenómeno, en el trabajo presentado en el **capítulo 3** se caracterizó la respuesta del mutante *pgi1-2* a los VCs emitidos por *A. alternata.* VCs emitidos por este hongo

fitopatógeno promovieron el crecimiento, la fotosíntesis y la acumulación de CKs plastidiales en hojas *pgi1-2*. Contra todo pronóstico, VCs emitidos por *A. alternata* promovieron la acumulación de niveles excepcionalmente elevados de almidón en hojas *pgi1-2*. Análisis proteómicos revelaron que los VCs microbianos promueven cambios en la acumulación de proteínas involucradas en la fotosíntesis, el metabolismo del almidón y el crecimiento que pueden explicar las respuestas observadas en plantas *pgi1-2*. Los datos presentados en este capítulo muestran que las plantas de Arabidopsis son capaces de responder a VCs microbianos mediante la activación o la estimulación de mecanismos independientes de pPGI.

SUMMARY

SUMMARY

Starch is a branched homopolymer of a-1,4-linked glucose subunits with a-1,6-linked glucose at the branching points. Synthesized in plastids, this reserve polysaccharide is the major storage carbohydrate in most higher plants and is an important determinant of plant growth and its relationship with the environment. It is widely accepted that the whole photosynthesis-driven starch biosynthetic process occurring in leaves resides exclusively in the chloroplast. According to this view, starch is considered the end-product of a metabolic pathway that is linked to the Calvin-Benson cycle (CBC) by means of the plastidic phosphoglucose isomerase (pPGI). This enzyme catalyzes the conversion of fructose-6-phosphate from the CBC into glucose-6-phosphate, which is metabolized into starch by the combined action of phosphoglucomutase, ADP-glucose pyrophosphorylase and starch synthase.

Plants are able to perceive biotic stimuli by recognizing a multitude of compounds originating from the interacting organisms. In this regard it is noteworthy that rhizosphere microorganisms synthesize a multitude of substances which regulate morphogenesis and plant growth. Moreover, these microbes emit many volatile compounds (VCs) that play potentially important roles as semiochemicals in the communication between plant and microorganism. Studies carried out by the research group where I have developed this doctoral thesis showed that VCs emitted by a wide range of microorganisms (including plant pathogens and microbes that do not normally interact with plants) promote accumulation of exceptionally high levels of starch in plants. Given the lack of knowledge about the mechanisms involved in this phenomenon and taking into account the important role that plays starch in the interaction of the plant with the environment, in this doctoral thesis, I investigated the molecular basis involved in the plant response to VCs, giving special attention to the role played by pPGI in this response.

The **First chapter** of this work describes the characterization of two mutants (*pgi1-2* and *pgi1*-3) lacking pPGI activity. Starch content in leaves in both mutants was 10-15% of that accumulated by wild type (WT) leaves. Contrary to what might be expected considering the classical view of starch biosynthesis, microscopy analyses revealed the presence of starch granules in the chloroplasts of mesophyll cells of these mutants. Both *pgi1-2* and *pgi1-3* displayed slow growth, reduced photosynthetic capacity phenotypes and a decrease of the NAD(P)H/NAD(P) ratio with respect to WT leaves.

Hormonomic analyses showed that the content of plastidic cytokinins (CKs) in *pgi1* leaves is exceedingly lower than in WT leaves. Noteworthy, exogenous application of CKs largely reverted the low starch content phenotype of *pgi1* leaves. The data presented in this work show that pPGI is an important determinant of photosynthesis, redox status, growth and starch accumulation in mesophyll cells likely as a consequence of its involvement in the production of oxidative pentose phosphate pathway/glycolysis intermediates necessary for the synthesis of plastidic CKs and reducing power.

Chapter 2, shows that VCs emitted by phylogenetically diverse microorganisms (including bacteria and fungi pathogens and nonpathogens) promote growth, accumulation of exceptionally high levels of starch and flowering of various plant species, including crops of agronomic interest. Moreover, Arabidopsis plants exposed to VCs emitted by the phytopathogen *Alternaria alternata,* showed enhanced of photosynthesis and accumulated high levels of CKs. The magnitude of this phenomenon was reduced in the mutant 35S:AtCKX1 with CK-deficiency and the CKs signaling *ahk2/3* mutant, providing evidence that CKs play essential roles in this phenomenon. Transcriptomic analyses of Arabidopsis leaves exposed to *A. alternata* VCs revealed changes in the expression of light- and CK-responsive genes involved in photosynthesis, flowering, growth and starch metabolism. Notably, many genes differentially expressed in plants treated with fungal VCs were also differentially expressed in plants exposed to VCs emitted by *Bacillus subtilis* GB03, suggesting that plants react to microbial VCs through activation/stimulation highly conserved regulatory mechanisms.

To better elucidate the mechanisms involved in the responses of plants to microbial VCs, and to investigate the extent to which pPGI is involved in this phenomenon, the work of **chapter 3** of the work is characterized the response of *pgi1-2* mutant to VCs emitted by *A. alternata.* VCs emitted by this fungal phytopathogen promoted growth, photosynthesis and accumulation of plastidic CKs in *pgi1-2* leaves. Notably, VCs emitted by *A. alternata* promoted accumulation of exceptionally high levels of starch in *pgi1-2* leaves thus challenging the "classic" interpretation of transitory starch biosynthesis. Proteomic analyses revealed that microbial VCs promote global changes in the expression of proteins involved in photosynthesis, starch metabolism and growth that account for the observed response in *pgi1-2* plants. The data presented in this chapter show that Arabidopsis plants are capable of responding to microbial VCs by activation/stimulation of pPGI independent mechanisms.

INTRODUCTION

Starch is the main storage carbohydrate in vascular plants. Its abundance as a naturally occurring compound of living terrestrial biomass is surpassed only by cellulose, and represents the primary source of calories in the human diet. Because starch is the principal constituent of the harvestable organ of many agronomic plants, its synthesis and accumulation also influences crop yields.

Synthesized in the plastids of both photosynthetic and non-photosynthetic cells, starch is an insoluble polyglucan produced by starch synthase (SS) using ADP-glucose (ADPG) as the sugar donor molecule. Starch has two major components, amylopectin and amylose, both of which are polymers of α-D-glucose units. Amylose is a linear polymer of up to several thousand glucose residues, whereas amylopectin is a larger polymer regularly branched with α-1,6-branch points. These two molecules are assembled together to form a semi-crystalline starch granule. The exact proportions of these molecules and the size and shape of the granule vary between species and between organs of the same plant. The diversity of both composition and physical parameters of starches from different botanical sources gives rise to their diverse processing properties and applications in both non-food sectors such as sizing agents in textile and paper industry, adhesive gums, biodegradable materials, etc., and in food industries, particularly in bakery, thickening, confectionary and emulsification (Slattery et al., 2000; Burrell, 2003; Mooney, 2009). Starch is also used as a feedstock for first generation bioethanol production (Goldemberg, 2007). From an industrial perspective, the utilization of starch as a cheap and renewable polymer and clean energy source is becoming increasingly attractive as a consequence of social concerns about industrial wastes generated from fossil fuels.

In general, the harvested parts of our staple crop plants are heterotrophic starch-storing organs. Worldwide annual starch production of cereal seeds (rice, maize, wheat, barley, etc.) is approximately 2000 million tones, whereas worldwide annual starch production of roots (e.g. cassava and taro) and tubers (e.g. potato and sweet potato) exceeds 700 million tones (Food and Agriculture Organization of the United Nations, http://faostat.fao.org), much of which are destined to non-food purposes. In 2012 for instance, 75 million tons of starch were extracted and used in many industrial applications (http://www.starch.dk/). The increasing demand of starch from non-food industries, the continuing rapid increase in the world´s population, predicted to reach 9 billion by 2050, and the growing loss of arable land to urbanization has spurred on efforts

to synthesize plants with higher starch content. Therefore, a thorough understanding of the mechanisms involved in starch metabolism and regulation is critically important for the rational design of experimental traits aimed at improving yields in agriculture, and producing more and better polymers that fit both industrial needs and social demands.

1. PROPOSED STARCH BIOSYNTHETIC PATHWAYS

Starch is found in the plastids of photosynthetic and non-photosynthetic tissues. Mature chloroplasts occurring in photosynthetically active cells possess the capacity of providing energy (ATP) and fixed carbon for the synthesis of starch during illumination. By contrast, production of long-term storage of starch taking place in amyloplasts of reserve organs such as tubers, roots and seed endosperms depends upon the incoming supply of carbon precursors and energy from the cytosol. This difference between the metabolic capacities of chloroplasts and amyloplasts has lead to the generally accepted view that the pathway(s) involved in starch production are different in photosynthetic and non-photosynthetic cells.

1.1. Starch biosynthesis in leaves

In leaves, a portion of the photosynthetically fixed carbon is retained within the chloroplasts during the day to synthesize starch, which is then remobilized during the subsequent night to support non-photosynthetic metabolism and growth by continued export of carbon to the rest of the plant. Due to the diurnal rise and fall cycle of its levels, foliar starch is termed “transitory starch”.

Transitory starch is a major determinant of plant growth (Sulpice et al., 2009), and its metabolism is regulated to avoid a shortfall of carbon at the end of the dark period. It is typically degraded in a near-linear manner during darkness, with only a small amount remaining at the end of the night. When changes in the day length occurs that leads to a temporary periods of carbon starvation, the carbon budget is rebalanced by increasing the rate of starch synthesis, decreasing the rate of starch breakdown and, consequently, decreasing the rate of growth during darkness. The importance of starch turnover in plant growth and biomass production is demonstrated by studies of mutants that are defective in starch synthesis and mobilization. These mutants grow at the same rate as wild-type (WT) plants under continuous light or during very long days, but show a large inhibition of growth in short days (Caspar et al., 1985; Lin et al., 1988; Gibon et al.,

2004), which is due to the transient depletion of carbon during the night.

Leaf starch mainly accumulates in the photosynthetic palisade and spongy mesophyll cells, although it can also be synthesized in epidermal cells, stomatal guard cells and in the bundle sheath cells surrounding the vasculature (Tsai et al., 2009). Various mechanisms have been proposed to describe starch synthesis and degradation in leaves. Given that starch breakdown has been thoroughly discussed in recent reviews (Streb and Zeeman, 2012), in the following lines we will focus on describing, analyzing and comparing the proposed mechanisms of starch synthesis in leaves.

1.1.1. A classic model of starch biosynthesis according to which the starch biosynthetic pathway is linked to the Calvin-Benson cycle by means of plastidial phosphoglucose isomerase

It is widely assumed that the whole starch biosynthetic process resides exclusively in the chloroplast and segregated from the sucrose biosynthetic process that takes place in the cytosol (Neuhaus et al., 2005; Streb et al., 2009; Stitt and Zeeman, 2012). According to this classical view of starch biosynthesis (which is schematically illustrated in **Figure 1**, starch is considered the end-product of a metabolic pathway that is linked to the Calvin-Benson cycle by means of the plastidial phosphoglucose isomerase (pPGI). This enzyme catalyzes the conversion of fructose-6-phosphate (F6P) from the Calvin-Benson cycle into glucose-6-phosphate (G6P), which is then converted into glucose-1-phosphate (G1P) by the plastidial phosphoglucomutase (pPGM). ADPG pyrophosphorylase (AGP) then converts G1P and ATP into inorganic pyrophosphate (PPi) and ADPG necessary for starch biosynthesis. These three enzymatic steps are reversible, but the last step is rendered irreversible upon hydrolytic breakdown of PPi by plastidial alkaline pyrophosphatase.

pPGI is strongly inhibited by light (Heuer et al., 1982) and by Calvin-Benson cycle intermediates such as erythrose-4-P and 3-phosphoglycerate (3PGA) (the latter being an indicator of photosynthetic carbon assimilation) that accumulate during the day at stromal concentrations higher than the Ki values for pPGI (Kelly and Latzko, 1980; Dietz, 1985; Backhausen et al., 1997; Zhou and Cheng, 2008). Although both these characteristics of pPGI, and the low stromal G6P/F6P ratio occurring during illumination (Dietz, 1985) would indicate that this enzyme is inactive during illumination (and thus not involved in transitory starch biosynthesis), genetic evidence showing that transitory starch

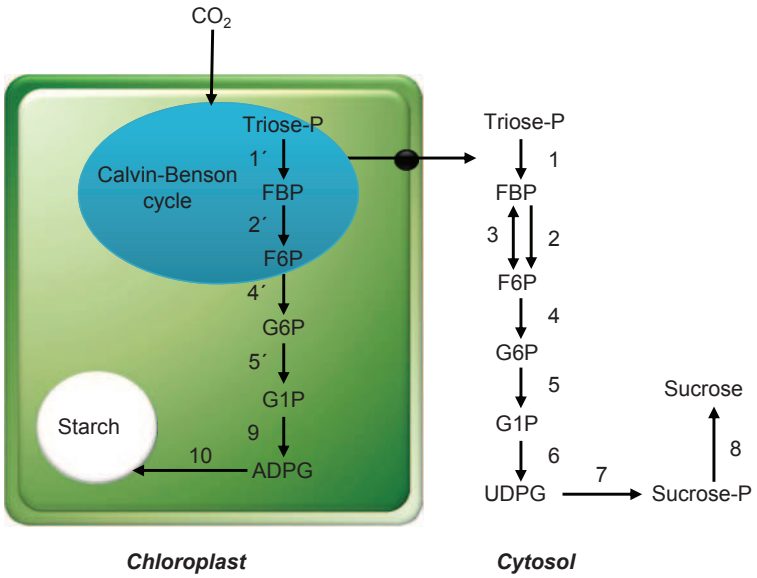

Figure 1. Classic interpretation of the mechanisms of starch and sucrose synthesis in leaves. The enzymes are numbered as follows: 1, 1´, fructose-1,6-bisphosphate aldolase; 2, 2´, fructose 1,6-bisphosphatase ; 3, PPi : fructose-6-P 1- phosphotransferase; 4, 4´, PGI; 5, 5´, PGM; 6, UGP; 7, sucrose-phosphate-synthase; 8, sucrose phosphate phosphatase; 9, AGP; 10, SS. According to this view, the starch biosynthetic process resides exclusively in the chloroplast and is segregated from the sucrose biosynthetic pathway that takes place in the cytosol.

biosynthesis occurs solely by the pPGI pathway has been obtained from characterization of starch-deficient pPGI mutants from various species (Jones et al., 1986; Kruckeberg et al., 1989; Yu et al., 2000; Niewiadomski et al., 2005; Kunz et al., 2010).

The classic view of leaf starch biosynthesis illustrated in **Figure 1** also implies that AGP is the sole source of ADPG, and functions as the major regulatory step in the starch biosynthetic process (Neuhaus et al., 2005; Streb et al., 2009; Stitt and Zeeman, 2012; Streb and Zeeman, 2012). This enzyme is a heterotetramer comprising two types of homologous but distinct subunits, the small (APS) and the large (APL) subunits (Crevillén et al., 2003, 2005). In Arabidopsis, six genes encode proteins with homology to AGP. Two of these genes code for small subunits (*APS1* and *APS2*, the latter being in a process of pseudogenization) and four (*APL1-APL4*) encode large subunits. Whereas APS1, APL1 and APL2 are catalytically active, APL3 and APL4 have lost their catalytic properties during evolution (Ventriglia et al., 2008). In Arabidopsis, the large subunits

are highly unstable in the absence of small subunits (Wang et al., 1998). Therefore, *APS1* T-DNA null mutants contain neither the large nor the small subunit proteins, which results in a total lack of AGP activity (Ventriglia et al., 2008; Bahaji et al., 2011).

AGP is allosterically activated by 3PGA, and inhibited by Pi (Kleczkowski, 1999). This property makes the production of ADPG highly sensitive to changes in photoassimilate (e.g. 3PGA) availability in the chloroplast, and helps to coordinate photosynthetic CO_2 fixation with starch synthesis in leaves. AGP activity is also subjected to APS redox regulation (Hendriks et al., 2003; Kolbe et al., 2005; Michalska et al., 2009; Li et al., 2012). Under oxidizing conditions the two APS subunits are covalently linked via an intermolecular disulfide bridge, thus forming a stable dimer within the heterotetrameric AGP enzyme (Fu et al., 1998; Hendriks et al., 2003). Conversely, under reductive conditions APS monomerization is accompanied by activation of AGP activity. APS dimerisation markedly decreases the activity of AGP and alters its kinetic properties, making it less sensitive to activation by 3PGA and increasing its K_m for ATP (Hendriks et al., 2003). In turn, APS monomerization activates AGP, which is then sensitive to fine control through allosteric activation by 3PGA.

By examining the correlation between APS redox status, AGP activity and starch levels in leaves, different works have provided evidence that transitory starch accumulation is finely regulated by APS post-translational redox modification in response to environmental inputs such as light, sugars and biotic stress (Hendriks et al., 2003; Gibon et al., 2004; Kolbe et al., 2005; Li et al., 2011). However, using *aps1* Arabidopsis plants ectopically expressing a redox-insensitive, mutated APS1 form, Li et al. (2012) and Hädrich et al. (2012) have independently shown that the rates of transitory starch accumulation in these plants are comparable to that of WT leaves. In addition, Li et al. (2012) reported WT rates of starch accumulation in *aps1* plants expressing in the chloroplast a redox-insensitive AGP from *Escherichia coli,* and concluded that post-translational redox modification of APS1 in response to light is not a major determinant of fine regulation of transitory starch accumulation in Arabidopsis leaves.

Plastidial NADP-thioredoxin reductase C (NTRC) plays an important role in protecting plants against oxidative stress (Serrato et al., 2004; Pulido et al., 2010). Serrato et al. (2004) and Michalska et al. (2009) reported that Arabidopsis *ntrc* mutants grown at 180 µmol photons sec^{-1} m^{-2} are small and their leaves accumulate low levels of starch when compared with WT leaves. Furthermore, Michalska et al. (2009) reported that *ntrc* leaves

exhibit a decrease in the extent of APS1 activation (reduction) by light. These authors thus concluded that NTRC is a major determinant of both light-dependent APS1 redox status and transitory starch accumulation. However, Li et al. (2012) have recently found that the size of Arabidopsis *ntrc* mutants is similar to that of WT plants when cultured at 90 µmol photons sec^{-1} m^{-2}. Under these conditions, APS1 redox status and starch accumulation rates in leaves of *ntrc* mutants are similar to those of WT leaves, strongly indicating that NTRC plays a minor role, if any, in the control of both light-dependent APS1 redox status and transitory starch accumulation in Arabidopsis cultured under non-stressing conditions.

In vitro assays using heterologously expressed potato and pea AGP have shown that APS can be activated by plastidial thioredoxins (Trxs) f and m (Ballicora et al., 2000; Geigenberger et al., 2005), indicating that these proteins could act as major determinants of APS redox status and of fine regulation of transitory starch accumulation. However, Li et al. (2012) found no differences in the rate of starch accumulation between WT leaves and leaves of homozygous *trxf*1*, trxf*2*, trxm*2 and *trxm*3 mutants. Furthermore, Thormählen et al. (2013) reported that starch levels in *trxf*1 leaves were only slightly reduced when compared with WT leaves. In addition, Sanz-Barrio et al. (2013) have shown that APS redox status in high-starch tobacco leaves overexpressing *Trxf* and *Trxm* genes from the plastid genome is similar to that of WT leaves. The overall data thus indicate that, individually, plastidial Trxs are not major determinants of APS1 redox status-controlled transitory starch content.

Genetic evidence showing that transitory starch biosynthesis occurs solely by the pPGI-pPGM-AGP pathway has been obtained from the characterization of leaves with reduced pPGM and AGP activity. For example, leaves of *APS1* and *pPGM* antisensed potato plants accumulate low levels of starch when compared with WT leaves (Müller-Röber et al., 1992; Lytovchenko et al., 2002; Muñoz et al., 2005). Furthermore, Arabidopsis *pgm* null mutants (Caspar et al., 1985; Kofler et al., 2000), *adg1-1* mutants with less than 3% of the WT AGP activity (Lin et al., 1988; Wang et al., 1998), and *APS1* T-DNA null mutants completely lacking AGP activity (Ventriglia et al., 2008; Bahaji et al., 2011) accumulate less than 2% of the WT starch. Further evidence supporting that AGP plays a crucial role in starch biosynthesis in leaves comes from alkaline pyrophosphatase-silenced plants of *Nicotiana benthamiana*, since their leaves accumulate ca. 60% of the WT starch (George et al., 2010).

1.1.2. Models of starch biosynthesis according to which the starch biosynthetic pathway is not linked to the Calvin-Benson cycle by means of plastidial phosphoglucose isomerase

A vast amount of biochemical and genetic data appear to support the starch biosynthetic pathway illustrated in **Figure 1** according to which (a) the whole starch biosynthetic process resides exclusively in the chloroplast, (b) pPGI links the Calvin-Benson cycle with starch biosynthesis, and (c) AGP is the sole source of ADPG linked to starch biosynthesis. However, in recent years an increasing volume of evidence has been provided that supports the occurrence of additional/alternative starch biosynthetic pathways involving the cytosolic and plastidial compartments wherein the supply of ADPG is not directly linked to the Calvin-Benson cycle by means of pPGI. Thus, Fettke et al. (2011) found that the envelope membranes of chloroplasts in mesophyll cells possess a yet to be identified G1P transport machinery enabling the incorporation of cytosolic G1P into the stroma, which is subsequently converted into starch by the stepwise AGP and SS reactions. According to these authors, such mechanism would only allow the accumulation of 1% of the WT starch, and would explain the occurrence of some trace amounts of starch in the pPGM mutants (Caspar et al., 1985; Muñoz et al., 2005; Streb et al., 2009).

Kunz et al. (2010) found that leaves of the *pgi1-2* null mutant impaired in pPGI activity accumulate 10% of the WT starch content, which is restricted to bundle sheath cells adjacent to the mesophyll and stomatal guard cells. This mutant exhibits high G6P transport activity as a consequence of the elevated expression of *GPT2* (Kunz et al., 2010), a gene that codes for a G6P/Pi translocator (GPT) mainly operating in heterotrophic tissues where the imported G6P can be used for the synthesis of starch and fatty acids or to drive the oxidative pentose phosphate pathway (Kang and Rawsthorne, 1996; Kammerer et al., 1998; Bowsher et al., 2007; Zhang et al., 2008a). According to Kunz et al. (2010) the unexpected high levels of starch occurring in leaves of pPGI mutants can be ascribed to high GPT2-mediated incorporation of G6P into the chloroplast of bundle sheath cells adjacent to the mesophyll and stomatal guard cells, where this hexose-phosphate can be then converted to starch by means of pPGM, AGP and SS as schematically illustrated in **Figure 2**. This interpretation, however, is hardly reconcilable with previous studies carried out by the same group showing that the starch-deficient phenotype of pPGI mutants can be totally reverted to WT starch

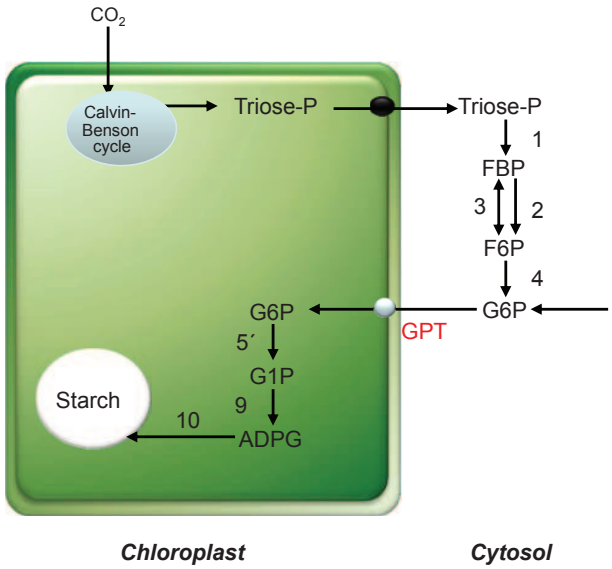

Figure 2. Suggested model of starch biosynthesis in bundle sheath cells adjacent to the mesophyll and stomatal guard cells of *pgi1-2* Arabidopsis mutants (from Kunz et al., 2010). Numbering of enzyme activities is the same as in **Figure 1.**

phenotype by the ectopic expression of GPT2 (Niewiadomski et al., 2005), and with the fact that leaves of the *pgi1-2/gpt2* double mutant accumulate as much as 60% of the starch occurring in *pgi1-2* leaves (Kunz et al., 2010). Furthermore, considering that (a) most of leaf starch accumulates in the mesophyll cells, (b) microscopic analyses revealed that stomatal guard cells and bundle sheath cells adjacent to the mesophyll of pPGI mutants accumulate nearly WT starch content (Tsai et al., 2009; Kunz et al., 2010), and (c) guard cells of WT leaves possess a G6P transport machinery (Overlach et al., 1993), it is difficult to understand how guard cells and bundle sheath cells adjacent to the mesophyll of pPGI mutants can accumulate as much as 10% of the WT starch as a consequence of high GPT2 activity. Needles to say, further investigations will be necessary to understand how and where *pgi* mutants accumulate ca. 10% of the WT starch.

Gerrits et al. (2001) have provided evidence that a sizable pool of sucrose in the plant cell has a plastidial localization. Consistent with the occurrence of a plastidial pool of sucrose, Murayama and Handa (2007) and Vargas et al. (2008) reported the presence of

a functional alkaline/neutral invertase (A/N-inv) inside plastids. Noteworthy, Vargas et al. (2008) reported that leaves of Arabidopsis mutants impaired in A/N-inv accumulate ca. 70% of the WT starch content. The same authors hypothesized that chloroplastic sucrose plays a sensing role in a mechanism of control of carbon partitioning and sucrose/starch ratio wherein glucose and fructose generated by A/N-inv are sensed through plastidial hexokinase (pHK) (Olsson et al., 2003; Giese et al., 2005). However, a possibility cannot be ruled out that, as schematically illustrated in **Figure 3**, plastidial sucrose acts as precursor for starch biosynthesis, its A/N-inv breakdown products being converted into starch by means of pHK, pPGM and AGP.

Sufficient evidence exists to support the view that a sizable pool of ADPG in leaves accumulates in the cytosol of photosynthetically competent cells. This hypothesis is compatible with the occurrence of cytosolic ADPG metabolizing enzymes such as ADPG phosphorylase (McCoy et al., 2006), glucan synthases (Tacke et al., 1991), sucrose synthase (SuSy) and ADPG hydrolases (Rodríguez-López et al., 2000; Olejnik and Kraszewska, 2005), and with the occurrence in the chloroplast envelope membranes of yet to be molecularly identified ADPG transport machineries whose activities can account for rates of starch accumulation occurring in leaves (Pozueta-Romero et al., 1991a). Genetic evidence supporting the occurrence of important ADPG source(s), other than AGP, has been obtained from different sources. First, leaves of transgenic potato and Arabidopsis plants ectopically expressing in the cytosol a bacterial ADPG hydrolase (Moreno-Bruna et al., 2001) accumulate lower ADPG levels than WT leaves, as determined by HPLC analyses (Baroja-Fernández et al., 2004; Bahaji et al., 2011). Second, ADPG content in the leaves of AGP and pPGM mutants is comparable to that of WT leaves, as confirmed by both HPLC (Muñoz et al., 2005; Bahaji et al., 2011) and HPLC:MS analyses (Baroja-Fernández et al., 2013). Third, HPLC:MS analyses of the triple *ss3/ss4/aps1* mutant impaired in AGP and SS class III and IV revealed that leaves from this mutant accumulate ca. 40-fold more ADPG than WT leaves (Ragel, 2012).

SuSy catalizes the reversible conversion of sucrose and NDP into fructose and the corresponding NDP-glucose, where N stands for uridine, adenosine, guanosine, cytidine, thymidine or inosine. Although UDP is generally considered to be the preferred nucleoside diphosphate for SuSy, ADP also serves as an effective substrate to produce ADPG (Delmer, 1972; Silvius and Snyder, 1979; Nakai et al., 1998; Zervosen et al., 1998; Porchia et al., 1999; Baroja-Fernández et al., 2003; Cumino et al., 2007;

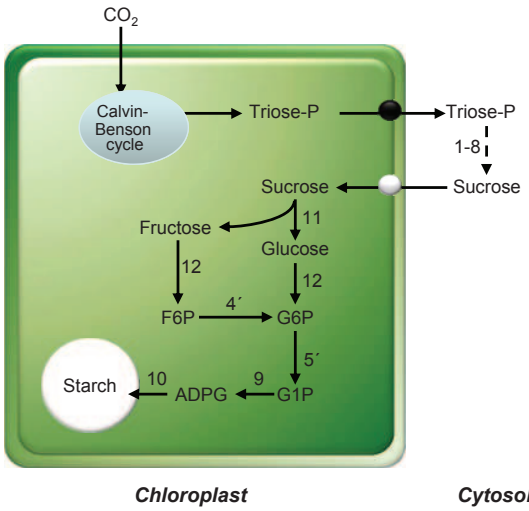

Figure 3. Suggested model of starch biosynthesis where sucrose entering the plastid is broken down by A/N-inv to produce fructose and glucose, the latter being converted to G6P by means of pHK. G6P is subsequently metabolized to starch by the stepwise reactions of pPGM, AGP and SS. Enzyme activities 1-10 and 4´ and 5´ are the same as in legend of **Figure 1.** Other enzymes involved are numbered as follows: 11, A/N-inv; 12, pHK.

Baroja-Fernández et al., 2012). SuSy is highly regulated at both transcriptional and post-transcriptional levels (Pontis et al., 1981; Koch et al., 1992; Fu and Park, 1995; Purcell et al., 1998; Déjardin et al., 1999; Asano et al., 2002; Ciereszko and Kleczkowski, 2002; Hardin et al., 2004; for a review see Kleczkowski et al., 2010). Although this sucrolytic enzyme is very active in reserve organs, it also expresses in the mesophyll cells of source leaves (Fu et al., 1995; Wang et al., 1999), its activity in potato and Arabidopsis leaves greatly exceeding the minimum needed to support normal rate of starch accumulation during illumination (Muñoz et al., 2005; Baroja-Fernández et al., 2012). Accordingly, a metabolic model of transitory starch biosynthesis has been proposed wherein (a) both sucrose and starch metabolic pathways are tightly interconnected by means of cytosolic ADPG producing enzymes such as SuSy (acting when cytosolic sucrose transiently accumulates during illumination), and by the action of an ADPG translocator located at the chloroplast envelope membranes, and (b) both AGP and pPGM play an important role in the scavenging of glucose units derived from starch breakdown occurring during starch biosynthesis and during the biogenesis of the starch granule (Baroja-Fernández et al., 2005; Muñoz et al., 2005, 2006; Bahaji et al., 2011) (**Figure 4**). Thus, the net

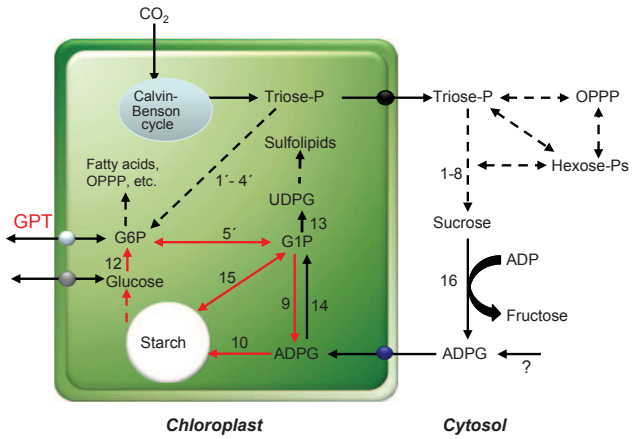

Figure 4. Suggested interpretation of the mechanism of starch biosynthesis in leaves according to which (a) ADPG is produced in the cytosol and transported to the chloroplast by the action of a yet to be identified ADPG translocator, (b) AGP and pPGM play an important role in the scavenging of glucose units derived from starch breakdown, and (c) starch metabolism is connected with other metabolic pathways through the metabolites generated by the starch futile cycle. Enzyme activities 1-10 and 1´-5´ are the same as in legend of **Figure 1**. Other enzymes involved are numbered as follows: 12, pHK; 13, plastidial UGP (Okazaki et al., 2009); 14, plastidial AGPP; 15, pSP (Zeeman et al., 2004); 16, SuSy. Starch to glucose conversion would involve the coordinated actions of amylases, isoamylases and disproportionating enzymes (Asatsuma et al., 2005; Kaplan and Guy, 2005; Fulton et al., 2008). Note that starch futile cycling (indicated with red arrows) may entail advantages such as rapid metabolic channeling toward various pathways (such as fatty acid (Periappuram et al., 2000), OPPP under stress conditions (Zeeman et al. 2004) and sulfolipid biosynthesis (Okazaki et al., 2009)) in response to physiological and biochemical needs. This view predicts that the recovery towards starch biosynthesis of the glucose units derived from the starch breakdown will be deficient in pPGM and AGP mutants, resulting in a parallel decline of starch accumulation and enhancement of soluble sugars content since starch breakdown derived products (especially glucose and G6P) will leak out the chloroplast through very active glucose translocator (Cho et al., 2011) and GPT2 (which is highly expressed in *pgi, pgm* and *agp* mutants, Kunz et al., 2010).

rate of starch accumulation is determined by the balance between the rates of SuSy-mediated ADPG synthesis in the cytosol, import of cytosolic ADPG to the chloroplast, starch synthesis, starch breakdown and by the efficiency with which starch breakdown products can be recycled back to starch via the coupled reactions of pPGM and AGP. Accordingly, this view predicts that the recovery towards starch biosynthesis of the glucose units derived from the starch breakdown will be deficient in pPGM and AGP mutants, resulting in a parallel decline of starch accumulation.

The occurrence of a substrate or "futile" cycle as a result of the simultaneous

synthesis and breakdown of starch in leaves is not surprising since pulse-chase and starch-preloading experiments using isolated chloroplasts (Stitt and Heldt, 1981; Fox and Geiger, 1984), intact leaves (Scott and Kruger, 1995; Walters et al., 2004), or cultured photosynthetic cells (Lozovaya et al., 1996) have shown that the illuminated chloroplasts can synthesize and mobilize starch simultaneously. Furthermore, enzymes involved in starch breakdown such as SEX1 and BAM1 are redox-activated during the light under different environmental conditions (Mikkelsen et al., 2005; Sparla et al., 2006; Valerio et al., 2010). Futile cycles lead to a net consumption of ATP, which in some instances can be estimated at about 60% of the total ATP produced by the cell (Hill and ap Rees, 1994; Alonso et al., 2005). Although these cycles appear to waste energy without any apparent physiological reason, they form part of the organization of the plant central metabolism, and contribute to its flexibility (Rontein et al., 2002). As presented above, starch acts as a major integrator in the plant growth that accumulates to cope with temporary starvation imposed by the environment (Sulpice et al., 2009). It is thus conceivable that highly regulated starch futile cycling may entail advantages such as sensitive regulation and rapid metabolic channeling toward various pathways in response to physiological and biochemical needs of the plant. Therefore, due to the importance of starch in the overall plant metabolism and growth, it is tempting to speculate that both redundancy of ADPG sources and starch futile cycling have been selected during plant evolution to warrant starch production and rapid connection of starch metabolism with other metabolic pathways.

The postulated starch biosynthetic pathways involving the plastidial and cytosolic compartments are consistent with still enigmatic results obtained from experiments carried out more than 50 years ago showing that ^{14}C in the glucose moiety of sucrose, starch, UDP-glucose and hexose-Ps is asymmetrically distributed in green leaves exposed to $^{14}CO_2$ for a short period of time (Kandler and Gibbs, 1956; Gibbs and Kandler, 1957; Havir and Gibbs, 1963). Unlike the "classic" view on transitory starch biosynthesis (**Figure 1**) predicting that green leaves exposed to $^{14}CO_2$ for a short period of time should synthesize starch with ^{14}C symmetrically distributed in the glucose moiety, the proposed mechanisms of starch synthesis illustrated in **Figures 2-4** provide a possible explanation for solving the above enigma. As shown in these figures, triose-Ps produced in the Calvin-Benson cycle are exported to the cytosol where they can be channeled into the oxidative pentose-P pathway (OPPP), thereby leading to a randomization of the

carbons giving rise to the asymmetric ^{14}C distribution observed in hexose-Ps, nucleotide-sugars and sucrose upon exposure to $^{14}CO_2$ for a short period of time. Cytosolic G1P, G6P, sucrose and/or ADPG will be then transported into the chloroplast and utilized as precursors for the synthesis of starch molecules possessing glucose molecules with asymmetric ^{14}C distribution.

Genetic evidence consistent with the occurrence of a link between sucrose and transitory starch metabolism can be obtained from sucrose-phosphate-synthase and cytosolic PGM deficient plants (Strand et al., 2000; Fernie et al., 2002), since their leaves contain low levels of both sucrose and starch as compared with WT leaves. Further genetic evidence can be obtained from sedoheptulose-1,7-bisphosphate overexpressing plants (Miyagawa et al., 2001; Lefebvre et al., 2005), since their leaves are characterized by having high levels of both sucrose and starch when compared with WT leaves. Finally, genetic evidence indicating that leaf sucrose and starch metabolic pathways are linked by SuSy has been obtained from SuSy-overexpressing potato plants whose leaves accumulate higher ADPG and starch contents than WT leaves (Muñoz et al., 2005). Further endeavors based on the characterization of mutants totally lacking SuSy will be necessary to confirm (or refute) the involvement of SuSy in starch biosynthesis in leaves.

1.2. Starch biosynthesis in heterotrophic organs

Sucrose produced in leaves is imported by the heterotrophic organs and used as carbon source for energy production and starch synthesis in the amyloplast. As in the case of leaves, various mechanisms have been proposed to describe starch biosynthesis in the heterotrophic organs, which together with the mechanisms of sucrose unloading, will be presented and discussed below.

1.2.1. Mechanisms of sucrose unloading in heterotrophic organs

Unloading of sucrose arriving from aerial parts of the plant into sink organs occurs either symplastically (moving via plasmodesmata) or apoplastically. The route depends on the species, organ or tissue. In the apoplastic delivery of sucrose to rapidely growing sinks, apoplastic sucrose that has been released into the extracellular space can be split by cell wall-bound invertases into glucose and fructose for subsequent uptake into the cell by hexose transporters. Alternatively, sucrose can be taken up by plasma membrane-

bound sink specific sucrose transporters of non-dividing storage sinks (Sauer, 2007). Sucrose is then transported via yet-to-be-identified tonoplast sucrose transporters to the vacuole, which serves as temporary reservoir. Apoplastic sucrose can also be taken up by a sucrose-induced endocytic process, and transported to the central vacuole of heterotrophic cells (Etxeberria et al., 2005a, 2005b; for a review see Etxeberria et al., 2012). Endocytic uptake of extracellular sucrose and subsequent transport to the vacuole is not in conflict with transport through membrane-bound carriers given that cell homeostasis can be better maintained if both mechanisms operate in parallel (Etxeberria et al., 2005a). Consistent with this view, Baroja-Fernández et al. (2006) reported that carrier-mediated sucrose import in starved cells of sycamore (*Acer pseudoplatanus* L.) plays an important role in the overall sucrose-starch conversion process during the initial period of sucrose unloading, whereas endocytosis may play an important role in transporting the bulk of sucrose to the vacuole and its subsequent conversion into starch after prolonged period of sucrose unloading.

1.2.2. Proposed mechanisms of sucrose-starch conversion in heterotrophic cells

Sucrose entering the heterotrophic cell by any of the mechanisms described above must be metabolized to molecules that can be transported to the amyloplast for subsequent conversion into starch. Because both chloroplasts and amyloplasts are ontogenically related, and because chloroplasts possess a very active triose-P translocator connecting the plastidial and cytosolic compartments, it was originally assumed that cytosolic triose-Ps entering the amyloplast by means of a triose-P translocator act as precursors for starch biosynthesis (Boyer, 1985). However, NMR spectroscopy experiments of starch biogenesis in wheat and maize grains using ^{13}C-NMR (Keeling et al., 1988; Hatzfeld and Stitt, 1990) demonstrated that a C-6 molecule, not a triose-P (C-3), is the precursor of starch biosynthesis in amyloplasts. Further investigations of the enzyme capacities of amyloplasts (Entwistle and ap Rees, 1988; Frehner et al., 1990) supported the view that C-6 molecules entering amyloplasts are the precursors of starch biosynthesis. Depending on the nature of the C-6 molecule entering the amyloplast (G6P or ADPG), and depending on the enzymes involved in ADPG synthesis, various mechanisms have been proposed to explain the sucrose-starch conversion process in heterotrophic cells.

1.2.2.1. Models of sucrose-starch conversion in heterotrophic cells according to which

AGP is the sole source of ADPG linked to starch biosynthesis

It is widely assumed that AGP is the sole source of ADPG in heterotrophic organs of mono- and di-cotyledonous plants. Maximal in vitro AGP activity greatly exceeds the minimum required to support the normal rate of starch accumulation in starch storing organs (Denyer et al., 1995; Weber et al., 2000; Li et al., 2013). This would indicate that AGP is not a rate-limiting step in the sucrose-starch conversion process in many heterotrophic organs. In fact, the flux control coefficient of AGP has been estimated to be as low as 0.08 in some heterotrophic organs (Denyer et al., 1995; Weber et al., 2000; Rolletschek et al., 2002). As presented above, AGP is a highly regulated enzyme. However, whereas evidence has been provided that starch synthesis is regulated by post-translational redox modification of AGP and 3PGA/Pi balance in potato tubers (Tiessen et al., 2003), AGP in developing barley, wheat, pea and bean seeds is insensitive to 3PGA and Pi regulation (Hylton and Smith, 1992; Kleczkowski et al., 1993; Weber et al., 1995; Gómez-Casati and Iglesias, 2002).

In heterotrophic organs of dicotyledonous plants, sucrose entering the cytosolic compartment of the heterotrophic cell is broken down by SuSy to produce fructose and UDPglucose (UDPG), the latter being converted to G1P and PPi by UDPG pyrophosphorylase (UGP). G1P is subsequently metabolized to G6P by means of the cytosolic phosphoglucomutase. Cytosolic G6P then enters the amyloplast, where it is converted to starch by the sequential activities of pPGM, AGP and SS (**Figure 5A**). Unlike chloroplasts, amyloplasts are unable to photosynthetically generate ATP and therefore, cytosolic ATP must enter the amyloplast to produce ADPG by means of AGP. The presence of an ATP/ADP translocator in the envelope membranes of amyloplasts has been firmly established by different laboratories (Pozueta-Romero et al., 1991b; Tjaden et al., 1998), and evidence about its relevance in the starch biosynthetic process has been provided by Tjaden et al. (1998) and Geigenberger et al. (2001) who reported that potato tubers with decreased plastidic ATP/ADP transporter activities exhibit reduced starch content.

Genetic evidence demonstrating the importance of SuSy in the sucrose-starch conversion process in heterotrophic organs of dicotyledonous plants comes from the reduced levels of starch in potato tubers and carrot roots exhibiting low SuSy activity (Zrenner et al., 1995; Tang and Sturm, 1999). In addition, genetic evidence showing that starch biosynthesis in heterotrophic organs involves the incorporation of G6P and

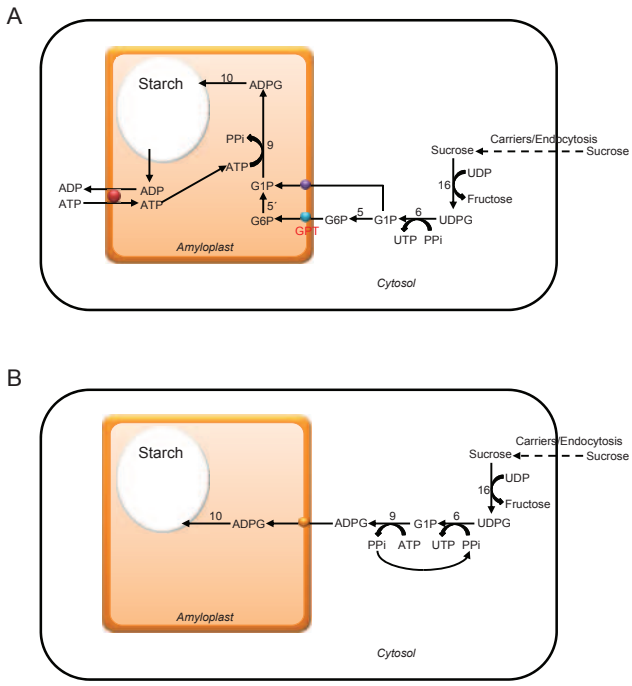

Figure 5. Classic interpretation of the mechanisms of sucrose-starch conversion in (A) heterotrophic cells of dicotyledonous plants and (B) cereal endosperm cells according to which AGP is the sole source of ADPG linked to starch biosynthesis. Note that in heterotrophic cells of dicotyledonous plants (a) AGP is exclusively localized in the plastid, (b) the cytosolic carbon substrate compound entering the amyloplast for subsequent conversion into starch is G6P, and (c) cytosolic UGP is involved in the sucrose-G6P conversion process. Plastidial and cytosolic G1P pools are likely connected by means of a yet to be identified G1P translocator occurring in amyloplasts (Fettke et al., 2010). Note also that in cereal endosperms cells (a) most of AGP has a cytosolic localization, and (b) the cytosolic carbon substrate compound entering the amyloplast for subsequent conversion into starch is ADPG. Numbering of enzyme activities is the same as in legends of **Figures 1-4.**

its subsequent conversion into ADPG by means of pPGM and AGP has been obtained from the characterization of *Vicia narbonensis* embryos expressing antisense *GPT* (Rolletschek et al., 2007) (these embryos, however, expressed lower levels of starch-related genes such as AGP and SuSy encoding genes), genetically engineered pea seeds and potato tubers with reduced pPGM (Harrison et al., 1998; Tauberger et al., 2000) and from *V. narbonensis* seeds and potato tubers with reduced AGP activity (Müller-Röber et al., 1992; Weber et al., 2000). These organs all exhibit reduced starch content when compared with their corresponding WT organs.

Using discs from WT and pPGM antisensed potato tubers incubated with radiolabelled G1P, Fettke et al. (2010) reported that G1P can be efficiently taken up by potato tuber parenchyma cells and converted to starch. Moreover, these authors reported that exogenously added G1P-dependent starch synthesis was diminished in tuber discs of potato plants with low plastidial starch phosphorylase (pSP) activity. Fettke et al. (2010) thus proposed that G1P can be taken up by amyloplast by a yet to be identified transporter to be subsequently used as substrate for pSP- and/or AGP-mediated starch biosynthesis. However, although this hypothesis can explain the results obtained using potato tuber discks, it conflicts with previous reports using pSP antisensed potato tubers showing that, *in planta*, the lack of pSP did not exert any effect on starch accumulation (Sonnewald et al., 1995). Furthermore, this hypothesis is hardly reconcilable with the strong reduction of starch content in tubers of pPGM antisensed potato tubers, indicating that plastidial G6P is linked to starch biosynthesis (Tauberger et al., 2000).

Unlike dicotyledonous plants where AGP is exclusively localized in the plastidial compartment, most of AGP in cereal endosperm cells has a cytosolic localization (Villand and Kleczkowski, 1994; Denyer et al., 1996; Kleczkowski, 1996; ThornbjØrnsen et al., 1996a, 1996b; Beckles et al., 2001; Johnson et al., 2003). Consistently, most of ADPG has a cytosolic localization in cereal endosperm cells (Liu and Shannon, 1981; Tiessen et al., 2012). Import studies using amyloplasts isolated from maize and barley endosperms of WT plants and starch-deficient/ADPG-excess maize *Zmbt1* and barley *Hvbt1* mutants provided genetic evidence that *Zea mays* brittle1 (BT1) (ZmBT1) and *Hordeum vulgare* BT1 (HvBT1) are membrane proteins that can incorporate cytosolic ADPG into the amyloplast (Liu et al., 1992; Shannon et al., 1996; Möhlmann et al., 1997; Shannon et al., 1998; Patron et al., 2004). Furthermore, cell fractionation studies revealed that most of ADPG accumulating in "high-ADPG" developing endosperms of the Riso 13 *Hvbt1* mutant has a cytosolic localization (Tiessen et al., 2012). The overall data thus indicate that, in cereal endosperm cells, BT1 proteins facilitate the transfer of cytosolic ADPG into the amyloplast for starch biosynthesis and are rate-limiting steps in this process. Based on this evidence, a starch biosynthesis model has been proposed for cereal endosperms wherein the stepwise reactions of SuSy, UGP and AGP take place in the cytosol to convert sucrose into ADPG, which enters the amyloplast by means of BT1 to be utilized as glucosyl donor for starch biosynthesis (**Figure 5B**) (Villand and Kleczkowski, 1994; Denyer et al., 1996). Genetic evidence

demonstrating the importance of SuSy in the sucrose-starch conversion process in cereal endosperms comes from QTL analyses in maize endosperms (Thévenot et al., 2005) and from the reduced levels of starch in maize seeds exhibiting low SuSy activity (Chourey and Nelson, 1976). Evidence showing the importance of AGP in starch biosynthesis in cereal endosperms comes from AGP mutants exhibiting reduced starch content (Tsai and Nelson, 1966; Johnson et al., 2003).

1.2.2.2. Models of sucrose-starch conversion in heterotrophic cells according to which both SuSy and AGP are involved in the synthesis of ADPG linked to starch biosynthesis

Sufficient evidence exists to support the view that heterotrophic organs possess in the cytosol important sources, other than AGP, of ADPG linked to starch biosynthesis. This hypothesis is compatible with the occurrence in the amyloplast envelope membranes of ADPG transporters (Pozueta-Romero et al., 1991b; Naeem et al., 1997; Shannon et al., 1998), and with the occurrence in heterotrophic organs of cytosolic ADPG metabolizing enzymes such as ADPG phosphorylase (Dankert et al., 1964; Murata, 1977; McCoy et al., 2006) and SuSy. As presented in section 1.1.2, SuSy is a highly regulated enzyme that can produce ADPG from sucrose and ADP. It plays a predominant role in determining both sink strength and phloem loading, and in the entry of carbon into metabolism in nonphotosynthetic cells. Maximum *in vitro* ADPG producing SuSy activity is particularly high in starch storing organs, being comparable to that of AGP in potato tubers (Baroja-Fernández et al., 2009) and 3-4 fold higher than that of AGP in developing barley and maize endosperms (Baroja-Fernández et al., 2003; Li et al., 2013). Furthermore, maximal ADPG producing SuSy activity in heterotrophic organs such as developing maize endosperm greatly exceeds the minimum required to support the normal rate of starch accumulation in this organ (Li et al., 2013), which would indicate that ADPG producing SuSy activity is not a rate limiting step in the sucrose-starch conversion process in heterotrophic organs.

Essentially in line with investigations describing the occurrence of simultaneous synthesis and breakdown of glycogen in heterotrophic bacteria and animals (David et al., 1990; Gaudet et al., 1992; Massillon et al., 1995; Bollen et al., 1998; Guedon et al., 2000; Montero et al., 2011), pulse chase as well as starch pre-loading experiments using isolated amyloplasts and heterotrophic organs have provided evidence about the occurrence of simultaneous synthesis and breakdown of starch in non-photosynthetic

cells of plants under conditions of active starch accumulation (Pozueta-Romero and Akazawa, 1993; Neuhaus et al., 1995; Sweetlove et al., 1996). The occurrence of starch futile cycling in heterotrophic organs has been further fortified by recent studies showing that global regulators of storage substance accumulation such as FLOURY ENDOSPERM2 (FLO2) positively co-regulates the expression of starch synthesis and breakdown genes during starch accumulation in developing rice seeds (She et al., 2010), and by the fact that down-regulation of starch breakdown functions in genetically engineered wheat and rice plants results in increased grain yield (Hakata et al., 2012; Ral et al., 2012). Furthermore, starch breakdown enzymatic activities are high in heterotrophic organs (Li et al., 2013). Consistently, "alternative/additional" models for the sucrose-starch conversion process in heterotrophic organs of dicotyledonous and monocotyledonous plants have been proposed according to which (a) cytosolic enzymes such as SuSy catalyze directly the *de novo* production from sucrose of ADPG, which is subsequently imported into the amyloplast by the action of an ADPG translocator, and (b) pPGM and AGP play an important role in the scavenging of the glucose units derived from the starch breakdown (**Figure 6)**. Accordingly, these models preview that the net rate of starch accumulation in heterotrophic cells is determined by the balance between the rates of ADPG synthesis in the cytosol, import of cytosolic ADPG to the amyloplast, starch synthesis and starch breakdown, and by the efficiency with which starch breakdown products can be recycled back to starch via the coupled reactions of pPGM and AGP. Also, these models predict that the recovery towards starch biosynthesis of starch breakdown products will be deficient in pPGM and AGP mutants, resulting in a parallel decline of starch accumulation.

The occurrence of the sucrose-starch conversion pathway illustrated in **Figure 6A** implies that neither UDPG produced by SuSy, nor cytosolic hexose-Ps derived from the action of UGP on UDPG, are involved in starch biosynthesis in heterotrophic organs of dicotyledonous plants. This view is consistent with the unexpected WT starch content phenotype of UGP antisensed potato tubers (Zrenner et al., 1993), and with the WT G6P and G1P contents of starch-deficient SuSy antisensed potato tubers (Baroja-Fernández et al., 2003). Also, this view is coherent with the unexpected starch-deficient phenotype of transgenic potato tubers heterologously expressing in the cytosol either yeast invertase plus glucokinase (Trethewey et al., 1998) or bacterial sucrose phosphorylase (Trethewey et al., 2001), since the high sucrolytic activity occurring in these tubers leads to drastic

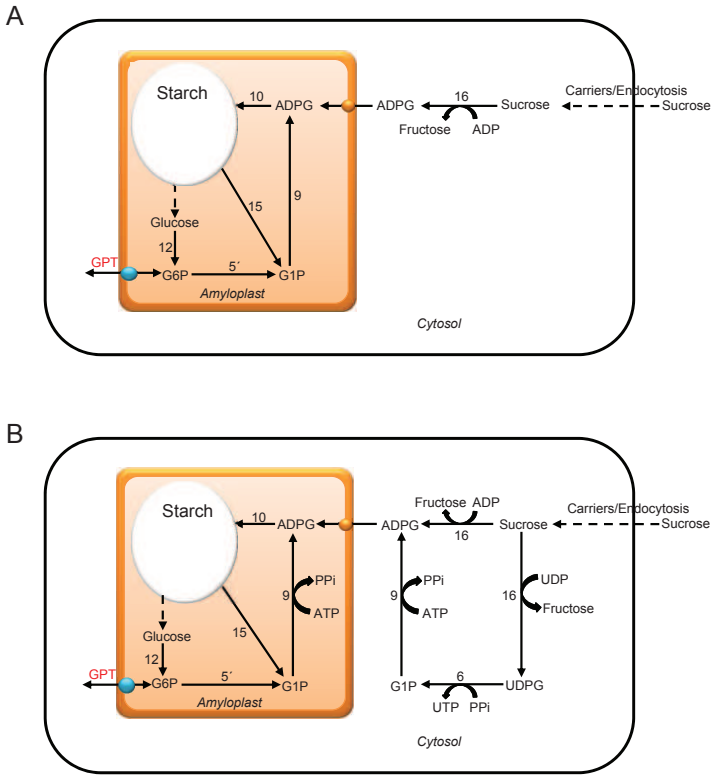

Figure 6. Suggested interpretation of the mechanisms of sucrose-starch conversion in (A) heterotrophic cells of dicotyledonous plants and (B) cereal endosperms according to which (a) both SuSy and AGP are involved in the synthesis of ADPG linked to starch biosynthesis, (b) the cytosolic carbon substrate compound entering the amyloplast for subsequent conversion into starch is ADPG, (c) pPGM and AGP are involved in the scavenging of starch breakdown products, and (d) the rate of starch accumulation would be the result of the net balance between starch synthesis and breakdown. Note that in heterotrophic cells of dicotyledonous plants neither cytosolic hexose-phosphates, nor cytosolic UGP are involved in the sucrose-starch conversion process. Note also that in cereal endosperm cells SuSy catalyzes the direct conversion of sucrose into both ADPG and UDPG, the latter being converted to ADPG in the cytosol by the stepwise reactions of UGP and AGP. This view predicts that in pPGM and AGP mutants the recovery towards starch biosynthesis of the glucose units derived from the starch breakdown will be deficient, resulting in a parallel decline of starch accumulation since starch breakdown products (especially glucose and G6P) will leak out the amyloplast. Numbering of enzyme activities is the same as in legends of **Figures 1-4.**

reduction of cytosolic sucrose levels, thus limiting ADPG-producing SuSy activity.

Genetic evidences demonstrating the significance of ADPG-producing SuSy in the sucrose-starch conversion process in heterotrophic cells of both mono- and di-

cotyledonous plants are the same as those presented in section 1.2.2.1.

2. MICROBIAL VOLATILES PROMOTE BOTH GROWTH AND ACCUMULATION OF EXCEPTIONALLY HIGH LEVELS OF STARCH IN MONO- AND DICOTYLEDONOUS PLANTS

Microbes synthesize and emit many volatile compounds (VCs). Volatile emissions from rhizobacterial isolates of beneficial *Bacillus* spp. promote growth in Arabidopsis plants by facilitating nutrient uptake, photosynthesis and defense responses, and by decreasing glucose sensing and abscisic acid levels (Ryu et al., 2003, 2004; Zhang et al., 2008b, 2009). In contrast, volatiles from *Pseudomonas* spp., *Serratia* spp. and *Stenotrophomonas* spp., and from some fungal species inhibit growth in Arabidopsis plants (Splivallo et al., 2007; Tarkka and Piechulla, 2007; Vespermann et al., 2007). Given the lack of knowledge on how microbial VCs affect reprogramming of carbohydrate metabolism in plants, Ezquer et al. (2010a) explored the effect on starch metabolism of volatiles released from different microbial species ranging from Gram-negative and Gram-positive bacteria to different fungi. Towards this end, the authors measured the starch and soluble sugars content in leaves of plants cultured in the presence or in the absence of adjacent microbial cultures. Noteworthy, Ezquer et al. (2010a) found that, in the absence of physical contact between plant and microbe, all microbial species tested (including plant pathogens and microbes that normally do not interact with plants) emitt volatiles that promote a rapid accumulation of exceptionally high levels of starch in leaves of both mono- and di-cotyledonous plants. Levels of starch reached by VCs-treated leaves were comparable to those occurring in heterotrophic organs such as tubers and cereal seeds. This phenomenon, initially designated as MIVOISAP (for MIcrobial VOlatiles Induced Starch Accumulation Process), was also accompanied by strong promotion of growth. Therefore, MIVOISAP cannot be ascribed to utilization of surplus photosynthates and/or ATP for starch biosynthesis that would occur under growth arrest conditions.

Transcriptome, metabolite content and enzyme activity analyses of potato leaves exposed to volatiles emitted by the plant pathogen *Alternaria alternata* revealed that starch over-accumulation was accompanied by enhanced 3PGA/Pi ratio and up-regulation of SuSy, acid invertase inhibitors, SSIII and SSIV, starch branching enzyme, GPT2, and synthesis of phosphatidylinositol-phosphates implicated in processes such as en-

docytosis and vesicle traffic (Ezquer et al., 2010a). MIVOISAP was also accompanied by down-regulation of acid invertase, plastidial Trxs, plastidial β-amylase and pSP, and proteins involved in the conversion of plastidial triose-phosphates into cytosolic G6P. No changes were found neither in the expression levels of AGP encoding genes nor in the redox status of APS1. Furthermore AGP-antisensed potato leaves accumulated exceptionally high levels of starch in the presence of VCs. The overall data thus indicated that potato MIVOISAP is the consequence of transcriptionally and post-transcriptionally regulated metabolic reprogramming involving (a) up-regulation of ADPG-producing SuSy, SSIII and SSIV, and proteins involved in the endocytic uptake and traffic of sucrose, and (b) down-regulation of acid invertase and starch breakdown enzymes. During potato MIVOISAP there also occurs a down-regulation of ATP-consuming functions involved in internal amino acid provision such as proteases and enzymes involved in de novo synthesis of amino acids. Ezquer et al. (2010a, 2010b) thus hypothesized that, under conditions of limited ATP-consuming protein breakdown occurring during exposure to VCs, the surplus ATP and carbon is diverted from protein metabolism to starch biosynthesis.

Time-course analyses of starch and maltose contents in illuminated Arabidopsis leaves of plants exposed to volatiles emitted by *A. alternata* revealed that both starch synthesis and β-amylase-dependent starch degradation were enhanced upon VCs treatment, although the final balance was positive for synthesis over breakdown (Li et al., 2011). The increase of starch content in illuminated leaves of VCs-treated *hy1/cry1*, *hy1/cry2* and *hy1/cry1/cry2* Arabidopsis mutants was many-fold lower than that of WT leaves, indicating that Arabidopsis MIVOISAP is subjected to photoreceptor-mediated control. Transcriptomic analyses of Arabidopsis leaves exposed to *A. alternata* volatiles revealed changes in the expression of genes involved in multiple processes. However, unlike potato, no changes could be observed in the expression of starch related genes (Li et al., 2011). Also, unlike potato plants, Arabidopsis MIVOISAP was accompanied by 2-3-fold increase of the levels of reduced (active) form of APS1. Using different Arabidopsis knockout mutants Li et al. (2011) observed that the magnitude of the VCs-induced starch accumulation was low in mutants impaired in SSIII, SSIV and NTRC. Unlike WT leaves, no changes in the redox status of AGP occurred in *ntrc* mutants, providing evidence that VCs-promoted monomerization (activation) of AGP is mediated by

NTRC. The overall data thus strongly indicated that Arabidopsis MIVOISAP involves a photocontrolled, transcriptionally and post-transcriptionally regulated network wherein photoreceptors and post-translational changes (e.g. changes in the redox status) of plastidial enzyme(s) such as AGP, SSIII and SSIV play important roles. Consistent with the possible involvement of changes in redox status of SSs in MIVOISAP, Glaring et al. (2012) have recently reported that SSI and SSIII are reductively activated enzymes.

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OBJECTIVES

OBJECTIVES

The studies on stimulatory effects of microbial VCs on plant growth and metabolism have been mainly focused on a few beneficial rhizosphere bacteria and fungi. To increase knowledge of microbial VCs-mediated interactions between plants and microorganisms in this thesis I explored the biochemical and molecular mechanisms involved in the response of plants to VCs emitted by phytopathogenic *A. alternata,* giving special attention to the role played by pPGI.

The **first objective** of this work was to study the role of pPGI in starch biosynthesis. The chapter 1 shows that pPGI is an important determinant of photosynthesis, energy status, growth and starch accumulation in mesophyll cells likely as a consequence of its involvement in the production of OPPP/glycolysis intermediates necessary for the synthesis of plastidic MEP-pathway derived hormones such as CKs, and not only the consequence of lack of pPGI-mediated flow between the Calvin-Benson cycle and the pPGM-AGP-SS starch biosynthetic pathway.

The **second objective** of this book was to investigate the biochemical and molecular mechanisms triggered in plants by VCs emitted by phylogenetically diverse microorganisms (including plant pathogens and microbes that do not normally interact mutualistically with plants). The overall data presented in this chapter show that VCs emitted by diverse phytopathogenic microorganisms promote plant growth, photosynthesis, starch accumulation, and flowering through cytokinin action.

The **objective of the third part** of this work was to explore the role of pPGI in the response of *Arabidopsis thaliana* to VCs. Data presented in this part of the thesis show that Arabidopsis plants are capable of responding to VCs emitted by phytopathogens by triggering pPGI independent mechanisms.

CHAPTER 1

Plastidic phosphoglucose isomerase is an important determinant of starch accumulation in mesophyll cells, growth, photosynthetic capacity, and biosynthesis of plastidic cytokinins in Arabidopsis

ABSTRACT

Phosphoglucose isomerase (PGI) catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate. It is involved in glycolysis and in the regeneration of glucose-6-P molecules in the oxidative pentose phosphate pathway (OPPP). In chloroplasts of illuminated mesophyll cells PGI also connects the Calvin-Benson cycle with the starch biosynthetic pathway. In this work we isolated *pgi1-3,* a mutant totally lacking pPGI activity as a consequence of aberrant intron splicing of the pPGI encoding gene, *PGI1*. Starch content in *pgi1-3* source leaves was ca. 10-15% of that of wild type (WT) leaves, which was similar to that of leaves of *pgi1-2,* a T-DNA insertion pPGI null mutant. Starch deficiency of *pgi1* leaves could be reverted by the introduction of a *sex1* null mutation impeding β-amylolytic starch breakdown. Although previous studies showed that starch granules of *pgi1-2* leaves are restricted to both bundle sheath cells adjacent to the mesophyll and stomata guard cells, microscopy analyses carried out in this work revealed the presence of starch granules in the chloroplasts of *pgi1-2* and *pgi1-3* mesophyll cells. RT-PCR analyses showed high expression levels of plastidic and extra-plastidic β-amylase encoding genes in *pgi1* leaves, which was accompanied by increased β-amylase activity. Both *pgi1-2* and *pgi1-3* mutants displayed slow growth and reduced photosynthetic capacity phenotypes even under continuous light conditions. Metabolic analyses revealed that the adenylate energy charge and the NAD(P)H/NAD(P) ratios in *pgi1* leaves were lower than those of WT leaves. These analyses also revealed that the content of plastidic 2-C-methyl-D-erythritol 4-phosphate (MEP)-pathway derived cytokinins (CKs) in *pgi1* leaves were exceedingly lower than in WT leaves. Noteworthy, exogenous application of CKs largely reverted the low starch content phenotype of *pgi1* leaves. The overall data show that pPGI is an important determinant of photosynthesis, energy status, growth and starch accumulation in mesophyll cells likely as a consequence of its involvement in the production of OPPP/glycolysis intermediates necessary for the synthesis of plastidic MEP-pathway derived hormones such as CKs.

INTRODUCTION

Starch is a branched homopolysaccharide of α-1,4-linked glucose subunits with α-1,6-linked glucose at the branched points. Synthesized by starch synthases (SS) using ADP-glucose (ADPG) as the sugar donor molecule, this polyglucan accumulates as predominant storage carbohydrate in plants. Starch is found in the plastids of photosynthetic and non-photosynthetic tissues. Mature chloroplasts occurring in photosynthetically active cells possess the capacity of providing energy (ATP) and fixed carbon for the synthesis of starch during illumination. By contrast, production of long-term storage of starch taking place in amyloplasts of reserve organs such as tubers, roots and seed endosperms depends upon the incoming supply of carbon precursors and energy from the cytosol. This difference between the metabolic capacities of chloroplasts and amyloplasts has lead to the generally accepted view that the pathway(s) involved in starch production are different in photosynthetic and non-photosynthetic cells (for a review see Bahaji et al., 2014b).

In leaves, up to 50% of the photosynthetically fixed carbon is retained within the chloroplasts during the day to synthesize starch (Stitt and Quick, 1989; Rao and Terry, 1995) which is then remobilized during the subsequent night to support non-photosynthetic metabolism and growth by continued export of carbon to the rest of the plant. Due to the diurnal rise and fall cycle of its levels, foliar starch is termed “transitory starch”. Many environmental factors such as photoperiod, light quality, senescence, temperature, contact with microorganisms, etc., influence transitory starch metabolism (Dreier et al., 1995; Kaplan and Guy, 2005; Ezquer et al., 2010; Valerio et al., 2010; Li et al., 2011). Because starch is a major integrator in the regulation of plant growth to cope with fluctuations in the carbon and energy status of the plant (Sulpice et al., 2009) the synthesis of this polyglucan in leaves is highly regulated at multiple levels in response to light and sugar signals and hormones such as cytokinins (CKs) (Werner et al., 2008; Erickson et al., 2014), abscisic acid (Ramon et al., 2007; Muñoz-Bertomeu et al., 2011) and brassinosteroids (Schlüter et al., 2002; Oh et al., 2011)

It is widely accepted that the whole photosynthesis-driven starch biosynthetic process occurring in mesophyll cells of leaves resides exclusively in the chloroplast (Arnon, 1955; Streb et al., 2009; Stitt and Zeeman, 2012). According to this classical view of starch biosynthesis, starch is considered the end-product of a metabolic pathway that is linked to the Calvin-Benson cycle by means of the plastidic phosphoglucose isomer-

ase (pPGI). This enzyme catalyzes the conversion of fructose-6-phosphate (F6P) from the Calvin-Benson cycle into glucose-6-phosphate (G6P), which is then converted into glucose-1-phosphate (G1P) by the plastidic phosphoglucomutase (pPGM). ADPG pyrophosphorylase (AGP) then converts G1P and ATP into inorganic pyrophosphate and ADPG necessary for starch biosynthesis. This view also implies that AGP is the sole source of ADPG, and functions as the major regulatory step in the starch biosynthetic process (Kleczkowski 1999, 2000; Streb et al., 2009; Stitt and Zeeman, 2012) However, despite the monumental amount of data supporting the classic interpretation of transitory starch biosynthesis in mesophyll cells, mounting evidence previews the possible occurrence of important additional pathway(s) involving the cytosolic and plastidic compartments (reviewed in Bahaji et al., 2014b).

In addition to its involvement in the connection of the Calvin-Benson cycle with the starch biosynthetic pathway in illuminated leaves, pPGI is involved in glycolysis and in the regeneration of G6P molecules in the oxidative pentose pathway (OPPP) in heterotrophic organs and non-illuminated leaves. pPGI is strongly inhibited by light (Heuer et al., 1982) and by 3-phosphoglycerate (3PGA) (Dietz, 1985) a Calvin-Benson cycle intermediate accumulating in the chloroplast during illumination that allosterically activates AGP (Kleczkowski, 1999, 2000). Although these characteristics of pPGI, and the low stromal G6P/F6P ratio occurring in the illuminated chloroplast (far lower than the equilibrium constant for pPGI (Dietz, 1985; Sharkey and Vassey, 1988) would indicate that this enzyme is inactive to some extent during illumination (and thus during transitory starch accumulation), genetic evidence showing that transitory starch biosynthesis occurs solely by the pPGI pathway has been obtained from characterization of starch-deficient mutants impaired in pPGI (Jones et al., 1986; Kruckeberg et al., 1989; Yu et al., 2000; Niewiadomski et al., 2005, Kunz et al., 2010). In Arabidopsis, such evidence has been obtained from the characterization of the *pgi1-1* and *pgi1-2* pPGI mutants accumulating up to 40% and 10% of the wild type (WT) starch content, respectively (Niewiadomski et al., 2005; Kunz et al., 2010). The *pgi1-1* allele has a single nucleotide substitution resulting in ca. 7% of the WT pPGI activity (Yu et al., 2000), whereas *pgi1-2* is a T-DNA insertion null mutant of *PGI1* that completely lacks pPGI activity (Kunz et al., 2010).

pgi1-2 leaves accumulate ca. 10-fold more starch than leaves impaired in pPGM and AGP (Caspar et al., 1985; Kofler et al., 2000; Ventriglia et al., 2008; Kunz et al., 2010;

Bahaji et al., 2011), which would apparently conflict with the widely accepted idea that the whole photosynthesis-driven starch biosynthetic process solely involves the Calvin-Benson cycle-pPGI-pPGM-AGP-SS pathway in mesophyll cells. However, consistent with the idea that pPGI-pPGM-AGP is the sole starch biosynthetic pathway operating in mesophyll cells, Kunz et al. (2010) showed that starch granules are restricted to both bundle sheath cells adjacent to the mesophyll and stomatal guard cells, and suggested that the occurrence of starch in these cells is due to the incorporation of cytosolic G6P into the chloroplast, where it is then metabolized into starch (Kunz et al., 2010).

During our searches for starch deficient plants of Arabidopsis we isolated and characterized a mutant, designated as *pgi1-3,* totally lacking pPGI activity as a consequence of the aberrant splicing of intron 6 of the pPGI encoding gene, *PGI1*. We found that, similar to *pgi1-2* leaves, starch content in *pgi1-3* leaves was ca. 10-15% of that of WT leaves. Contrary to expectations, microscopy analyses carried out in this work revealed the presence of starch granules in the mesophyll cells of the two *pgi1* mutants. Subsequent biochemical characterization of *pgi1* plants showed that pPGI is an important determinant of photosynthesis, energy status, growth and starch accumulation in mesophyll cells likely as a consequence of its involvement in the production of OPPP/glycolysis intermediates necessary for the synthesis of plastidic 2-C-methyl-D-erythritol 4-phosphate (MEP)-pathway derived hormones such as CKs. The data also support the occurrence in mesophyll cells of important pPGI independent starch biosynthetic pathway(s) involving the cytosolic and chloroplastic compartments.

MATERIAL AND METHODS

Plants, growth conditions and sampling

The work was carried out using *Arabidopsis thaliana* L. (Heynh) (ecotypes Col-*0* with erecta-105 mutation (*er-105*) , Col-*0* and Ws-2), the NASC N92274 (*pgi1-3*), the *pgi1-2* mutant (Kunz et al., 2010), the *aps1::T-DNA* mutant (SALK_040155), the *pgm::T-DNA* mutant (GABI_094D07), the *gpt2::T-DNA* mutant (GABI_454H06), *pgi1-3* plants expressing either *PGI1* from WT plants or *PGI1** from *pgi1-3* plants, the *pgi1-3/gpt2* and *pgi1-2/gpt2* double mutants and the *pgi1-2/sex1* and *pgi1-3/sex1* double mutants. The *pgi1-2/sex1* and *pgi1-2/gpt2* double mutants were confirmed by PCR using the oligonucleotide primers listed in **Table 1**. The *35S-PGI1* and *35S-PGI1** plasmid

constructs utilized to produce *PGI1* or *PGI1** expressing *pgi1-3* plants were produced as illustrated in **Supplemental Figure 1**. *pgi1-3* plants expressing granule bound starch synthase fused with green fluorescent protein (GBSS-GFP) were produced using the *35S-GBSS-GFP* plasmid construct (Szydlowskiet al., 2009) whereas *pgi1-2* plants expressing GBSS-GFP were produced using the *35S-GBSS-GFP** plasmid construct produced as illustrated in **Supplemental Figure 2**. The plasmid constructs were transferred to *Agrobacterium tumefaciens* EHA105 cells by electroporation and utilized to transform Arabidopsis plants according to Clough and Bent (1998). Transgenic plants were selected on the appropriate antibiotic-containing selection medium.

Unless otherwise indicated plants were cultured in soil in growth chambers under the indicated photoperiodic conditions (light intensity of 90 µmol photons sec^{-1} m^{-2}) and at a constant temperature of 22ºC. Harvested source leaves were immediately freeze-clamped and ground to a fine powder in liquid nitrogen with a pestle and mortar. To analyze the effects of exogenously applied CKs on starch content plants were grown *in vitro* on MS agar plates at a constant temperature of 22ºC under long day (LD) (16 h light/8 h dark) conditions. Three-weeks old plants were then transferred to MS agar plates containing the indicated concentrations of *trans*-zeatin (tZ). After two additional days leaves were harvested, and starch content was measured as described below.

Table 1. Primers used to identify *pgi1-2/sex1* and *pgi1-2/gpt2* mutants by PCR

Mutant	Designation	Sequence
pgi1-2	Forward	5'-TATACTCTTCTTCTCCATCTCTCAAAC-3'
	Reverse	5'-CTTTTAATCAGAAAAACCTAAGAGAGG-3'
	T-DNA	5'-CATTTTATAATAACGCTGCGGACATCTAC-3'
sex1	Forward	5'-GTCAGTCTATCCTGCGCTTTG-3'
	Reverse	5'-TCCGGTATGACAAGTCGAATC-3'
	T-DNA	5'-GCGTGGACCGCTTGCTGCAACT-3'
gpt2	Forward	5'-CTTCATGGGAGAGACTTTCCC-3'
	Reverse	5'-TGATCTCACCGGAATGTTCTC-3'
	T-DNA	5'-CCCATTTGGACGTGAATGTAGACAC-3'

Enzyme assays

One g of the frozen powder (see above) was resuspended at 4ºC in 3 ml of 100 mM HEPES (pH 7.5), 2 mM EDTA and 2 mM dithiothreitol, 1 mM PMSF and 10 ml/L protease inhibitor cocktail (Sigma P9599), and centrifuged at 14,000 x g for 20 min. The supernatant was desalted by ultrafiltration on Vivaspin 500 centrifugal concentrator (Sartorius) and the protein extract thus obtained was assayed for enzymatic activities. AGP and UDP-glucose (UDPG) pyrophosphorylase (UGP) activities were measured following the two-step assay method described in Li et al. (2012). PGI and sucrose synthase (SuSy) were measured as described in Jones et al. (1986) and Baroja-Fernández et al. (2102), respectively. Adenylate kinase was assayed in the two directions of the reaction as described in Kleczkowski and Randall (1986) using an HPLC system (Waters corporation) fitted with a Partisil 10-SAX column. PGM and acid invertase were assayed as described in Caspar et al. (1985) and Baroja-Fernández et al. (2009), respectively. ribulose 1,5-bisphosphate carboxylase oxygenase (Rubisco) activity was measured according to Pérez et al. (2005). Amylolytic activities were assayed as described in Liu et al. (2005). Alkaline pyrophosphatase (PPase) and sucrose-phosphate synthase (SPS) were measured as described in Linden et al. (1975). Starch synthase (SS) activity was measured in two steps: (1) SS reaction and (2) measurement of ADP produced during the reaction. The SS assay mixture contained 50 mM HEPES (pH 7.5), 6 mM $MgCl_2$, 3 mM dithiothreitol, 1 mM ADPG and 3% glycogen. After 5 min at 37ºC reactions were stopped by boiling the assay mixture for 2 min. ADP was measured by HPLC on a Waters Associate´s system fitted with a Partisil-10-SAX column. One unit (U) is defined as the amount of enzyme that catalyzes the production of 1 µmol of product per min.

Non-reducing western blot analyses of AGP

For non-reducing western blots of AGP, 50 mg of the homogenized frozen material (see above) was extracted in cold 16% (w/v) TCA in diethyl ether, mixed, and stored at –20ºC for at least 2 h as described in Li et al. (2012). The pellet was collected by centrifugation at 10,000 x g for 5 min at 4ºC, washed 3 times with ice-cold acetone, dried briefly under vacuum, and resuspended in 1x Laemmli sample buffer containing no reductant. Protein samples were separated on 10% SDS-PAGE, transferred to nitrocellulose filters, and immunodecorated by using antisera raised against maize AGP as primary antibody

Li et al. (2012), and a goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma) as secondary antibody.

Chromatographic separation of cytPGI and pPGI

Chromatographic separation of the two PGI isoforms was conducted using an AKTA FPLC from Amersham Pharmacia Biotech. Protein extracts of WT and *pgi1-3* leaves (see above) were loaded onto a HiLoad 16/10 Q-sepharose HP anion exchange column (Amersham Pharmacia Biotech) equilibrated with 50 mM HEPES (pH 7.5). After washing the column, the adsorbed proteins were eluted with a linear 0-0.8 M NaCl gradient in 50 mM HEPES (pH 7.5). The flow rate was 5 ml/min and 2.5 ml fractions were collected. Fractions were analyzed for PGI activity as described above.

Native gel assay for PGI activity

PGI zymograms were performed as described in Caspar et al. (1985). Protein extracts (see above) of both WT and *pgi1* leaves were loaded onto a 7.5% (w/v) polyacrylamide gel. After electroforesis gels were stained by incubating in darkness at room temperature with 0.1 M Tris-HCl (pH 8.0), 5 mM F6P, 1 mM NAD^+, 4 mM $MgCl_2$, 0.2 mM methylthiazolyldiphenyl-tetrazolium bromide (Sigma M5655) and 0.25 mM phenazine methosulfate (Sigma P9625) and 1 U/mL of G6P dehydrogenase from *Leuconostoc mesenteroides* (Sigma G8404).

Analytical procedures

For determination of metabolites content, fully expanded source leaves of 30 days after sowing (DAS) plants were harvested at the indicated illumination period, freeze-clamped and ground to a fine powder in liquid nitrogen with a pestle and mortar. ADPG content was measured by HPLC-MS/MS as described in Bahaji et al. (2014a). For measurement of sucrose, glucose and fructose, a 0.1 g aliquot of the frozen powder was resuspended in 1 mL of 90% ethanol, left at 70ºC for 90 min and centrifuged at 13,000 x g for 10 min. For measurement of G6P, F6P and G1P 0.5 g aliquot of the frozen powdered tissue was resuspended in 0.4 ml of 1 M $HClO_4$, left at 4ºC for 2 h and centrifuged at 10,000 x g for 5 min. The supernatant was neutralized with K_2CO_3 and centrifuged at 10,000 x g. Sucrose, glucose, fructose, F6P, G6P and G1P from supernatants were determined by HPLC with pulsed amperometric detection on a DX-500 Dionex system. NADP(H)

and NAD(H) were measured as described in Queval and Noctor (2007). Starch was measured by using an amyloglucosydase–based test kit (Boehringer Mannheim, Germany). For measurement of adenine nucleotides a 0.5 g aliquot of the frozen powder was resuspended in 0.4 ml $HClO_4$, left at 4ºC for 2 h and centrifuged at 10,000 x g for 5 min. The supernatant was neutralized with K_2CO_3 and centrifuged at 10,000 x g. Nucleotides content in the supernatant was measured by HPLC (Waters corporation) fitted with a Partisil 10-SAX column as described in Sweetlove et al. (1996). Recovery experiments were carried out by the addition of known amounts of metabolites standards to the frozen tissue slurry immediately after addition of extraction solutions.

For determination of CKs levels, aliquots of the frozen leaves (see above) were lyophilized and CKs were quantified according to the method described in Novák et al. (2008).

Iodine staining

Leaves harvested at the end of the light period were fixed by immersion into 3.7% formaldehyde in phosphate buffer. Leaf pigments were then removed in 96% ethanol. Re-hydrated samples were stained in iodine solution (KI 2% (w/v) I_2 1% (w/v)) for 30 min, rinsed briefly in deionized water and photographed.

Gas exchange determinations

Fully expanded apical leaves were enclosed in a LI-COR 6400 gas exchange portable photosynthesis system (LI-COR, Lincoln, Nebraska, USA). The gas exchange determinations were conducted at 25ºC with a photosynthetic photon flux density of 350 µmol m^{-2} s^{-1}. Net photosynthetic CO_2 fixation rates (A_n) was calculated using equations developed by von Caemmerer and Farquhar (1981). Stomatal conductance (g_s) values were determined as described in Harley et al. (1992). From the A/intercellular CO_2 concentrations (*Ci*) curves, maximum carboxylation rate (*Vcmax*), triose phosphate use (TPU) and maximum rate of the electron transport (*Jmax*) were calculated according to Long and Bernacchi (2003). To avoid miscalculation of A_n and *Ci* due to leakage into the gasket of the gas analyzer, we performed CO_2 response curves using an empty chamber. The values obtained for A_n and *Ci* in the empty chamber were compared with those of the chamber filled with a leaf and substracted from the values obtained with the empty chamber. The photosynthetic electron transport rate (ETR) values were calculated

according to Krall and Edwards (1992) as photosystem II (PSII) operating efficiency (Φ_{PSII}) x PPFD x 0.84 x 0.5, where PPFD is the photosynthetic photon flux density incident on the leaf, 0.5 was used as the fraction of excitation energy distributed to PSII (Ögren and Evans, 1993) and 0.84 as the fractional light absorbance (Morales et al., 1991). The rate of mitochondrial respiration in the dark was determined by measuring the rate of CO_2 evolution in the dark.

Real-time quantitative PCR

Total RNA was extracted from leaves using the trizol method according to the manufacturer´s procedure (Invitrogen). RNA was treated with RNAase free DNAase (Takara). 1.5 µg RNA was reverse transcribed using polyT primers and the Expand Reverse Transcriptase kit (Roche) according to the manufacturer's instructions. Real time quantitative PCR reaction was performed using a 7900HT sequence detector system (Applied Biosystems) with the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. Each reaction was performed in triplicate with 0.4 µL of the first strand cDNA in a total volume of 20 µL. The specificity of the PCR amplification was checked with a heat dissociation curve (from 60ºC to 95ºC). Comparative threshold values were normalized to 18S RNA internal control. The specificity of the obtained RT-PCR products was controlled on 1.8% agarose gels. Primers used for RT-PCRs of *PGI1,* β-amylase (*BAM)1, BAM2, BAM3* and *BAM5* are listed in **Table 2**.

Confocal microscopy

Subcellular localization of GFP-tagged GBSS was performed using D-Eclipse C1 confocal microscope (NIKON, Japan) equipped with standard Ar 488 laser excitation, BA515/30 filter for green emission and BA650LP filter for red emission.

Light and electron microscopy

Light microscopy and transmission electron microscopy (TEM) analyses were carried out essentially as described in Szydlowski et al. (2009). Briefly, small pieces (2 mm^2) of leaves were immediately fixed by submersion in a solution of 3% glutaraldehyde (v/v) in 0.05 M sodium cacodylate buffer, pH 7.4 (3 h at 4ºC, under vacuum). After fixing, the specimens were washed in a cacodylate buffer (0.05 M sodium cacodylate, 1% sucrose),

Table 2. Primers used in Real Time PCR

Gene	Direction	Sequence
18S RNA	Forward	5´-GGGCATTCGTATTTCATAGTCAGAG-3´
At3g41768	Reverse	5´-CGGTTCTTGATTAATGAAAACATCCT-3´
pPGI	Forward	5´-GGGATTAATGTTAGGGAGATGC-3´
At4g24620	Reverse	5´-TGTTACCGTCAAGATCAAACTC-3´
BAM1	Forward	5´-CTTGATCAAAACAATGAGGGAG-3´
At3g23920	Reverse	5´-CTTCCCGACATCTATGTGAG-3´
BAM2	Forward	5´-ACCGATCCTGATGGTCGCC-3´
At4g00490	Reverse	5´-CTCTTGTGTCCCTGGAGCC-3´
BAM3	Forward	5´-GATTCTGGAAATGGGTTAACC-3´
At4g17090	Reverse	5´-GTCACTTCCAGTTGTGTCTTC-3´
BAM5	Forward	5´-CACTACGGCATTCTCAACTTC-3’
At4g15210	Reverse	5´-CCTTTGGCTCCATAGGTCTC-3’

three times for 30 min each at 4ºC, and post-fixed with a solution of 1% osmium tetroxide in the above cacodylate buffer (overnight, 4ºC). After two washes, 30 min each, at 4ºC with the same cacodylate buffer, the samples were dehydrated in an ethanol series and progressively embedded in LR White resin (London Resin Co., Reading, UK). Semithin (1 µm) sections were stained with 1% (w/v) toluidine blue in aqueous 1% sodium borate for direct observation with a Zeiss Axiophot photomicroscope (Zeiss, Oberkochen, Germany). Ultrathin (70-90 nm) sections for TEM were constructed with 2% aqueous uranyl acetate and lead citrate. Observations were performed with a STEM LEO 910 electron microscope (Oberkochen, Germany) at 80 kV, equipped with a Gatan Bioscan 792 camera (Gatan, Pleasanton, CA, USA).

Statistical analysis

The data presented are the means of three independent experiments, with 3-5 replicates for each experiment (means ± SE). The significance of differences between the control

and the transgenic lines was statistically evaluated with Student´s t-test using the SPSS software. Differences were considered significant at a probability level of $P<0.05$. In CKs analyses, significance was determined by one-way univariate analysis of variance (ANOVA) for parametric data and Kruskal Wallis for non-parametric data, using the open source R software 2.15.1 (http://cran.r-project.org/). Multiple comparisons after ANOVA were calculated using the post hoc Tukey's honestly significant difference (HSD) test.

RESULTS

Identification and molecular characterization of a new pPGI null allele

Data available from the Arabidopsis Information Resource (http://www.arabidopsis.org) on the pPGI encoding *At4g24620* gene (*PGI1*) includes the Cs92274 *PGI1* polymorfism occurring in ethyl methanesulfonate-mutagenized plants in the *er-105* background. To identify its mutations site(s) we cultured plants from N92274 seeds obtained from the Nottingham Arabidopsis Stock Centre (NASC), and sequenced the *PGI1* gene.

PGI1 contains 14 exons interrupted by 13 introns (**Figure 1A**) (Yu et al., 2000). Sequencing analyses revealed that the pPGI N92274 allele harbors a G to A transition in the UUCA**G**/AU sequence of the 3´splice donor site of intron 6 (**Figure 1B,C**). To investigate whether this mutation would cause mis-splicing of the *PGI1* pre-mRNA in N92274, we amplified by reverse transcripion (RT)-PCR the *PGI1* mRNA from both WT and the N92274 leaves. Sequencing of the resulting complete cDNAs revealed that the cDNA obtained from N92274 leaves lacks 6 nucleotides (ATCAAG) downstream the single point mutation site (**Supplemental Figure 3**). The overall data thus showed that (i) the N92274 mutation leads to aberrant splicing of intron 6 during *PGI1* pre-mRNA maturation (**Figure 1C**), resulting in the production of a 6 nucleotides shorter *PGI1* mRNA, (ii) the 3´ splicing site of intron 6 of N92274 *PGI1* pre-mRNA occurs in a non-canonical UCAAG/AA sequence located 6 nucleotides downstream the WT 3´splicing site of *PGI1* pre-mRNA, and (iii) N92274 *PGI1* encodes a protein that lacks two amino acids (Ile346-Lys347) occurring in the WT pPGI (**Figure 1C, Supplemental Figure 4**).

As a first step to investigate whether the above mutation affects pPGI activity we measured the starch content in leaves of N92274 plants and its corresponding WT (*er-*

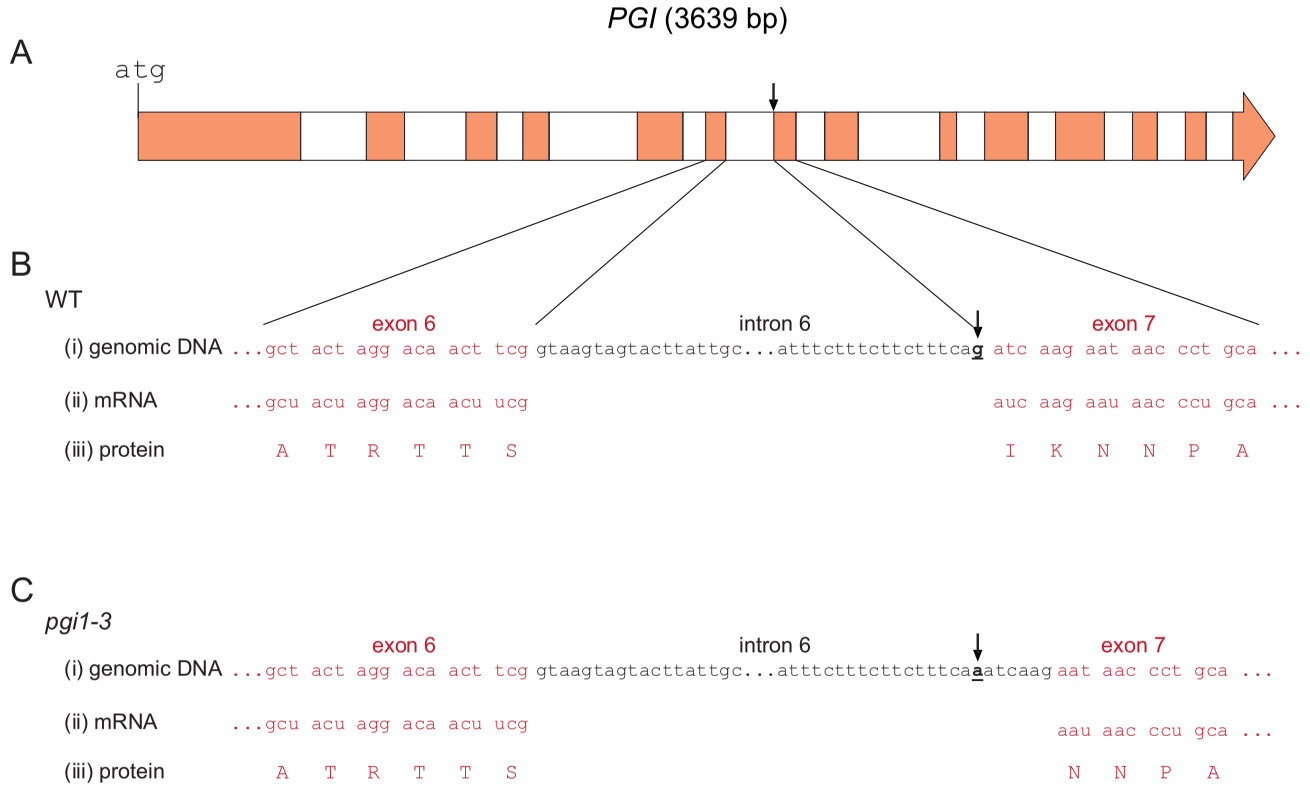

Figure 1. Molecular characterization of *pgi1-3*. (A) Genomic *PGI1* structure. In "B" and "C", comparisons between the genomic DNA (i), the mRNA (ii), and the derived protein sequence (iii) of WT and the N92274 (*pgi1-3*) mutant. In "i" only the splicing sequences between the exon 6 and intron 6, and between the intron 6 and exon 7 are shown. Arrows indicate the point mutation site in *pgi1-3*. Exon sequences and their respective deduced amino acidic sequences are highlighted in red color.

105) plants. As reference, we also measured the starch content in the leaves of *pgi1-2* T-DNA insertion mutant totally impaired in pPGI activity (Kunz et al., 2010) and its corresponding WT Wasilewskija (Ws-2) plants. Preliminary iodine staining analyses of leaves revealed that both N92274 and *pgi1-2* display a pale brown stain phenotype (**Figure 2A**). This phenotype contrasts with the dark brown staining phenotype of WT leaves and the yellow stain phenotype of the near starch-less *aps1* and *pgm* leaves impaired in AGP and pPGM, respectively (**Figure 3**). This indicated the presence of reduced starch content in both N92274 and *pgi1-2* leaves. In line with this presumption, quantitative starch content measurement analyses revealed that starch content in both N92274 and *pgi1-2* leaves was ca. 10-15% of that accumulated by WT leaves (**Figure 2B**).

The above results indicated that the mutation in the pPGI N92274 allele (thereafter designated as *pgi1-3*) totally abolishes pPGI activity. To test this hypothesis we measured total PGI activity in *pgi1-3* plants. We also carried out zymogramic and Q-sepharose chromatographic analyses of PGI activity as described in Materials and Methods. Two PGI isozymes exist in Arabidopsis, one in the plastids and the other in the cytosol (Caspar et al., 1985; Kofleret al., 2000; Yu et al., 2000; Niewiadomskiet al., 2005; Ventriglia et al., 2008; Tsai et al., 2009; Kunz et al., 2010; Bahaji et al., 2011). Typically, pPGI activity constitutes ca. 20-30% of the total cellular PGI (Jones et al., 1986; Kruckeberg et al., 1989; Yu et al., 2000; Niewiadomski et al., 2005, Kunz et al., 2010). Similarly to leaves of the *pgi1-2* T-DNA insertion mutant (Kunz et al., 2010), total PGI activity in *pgi1-3* leaves (928 ± 52 mU/g FW) was ca. 80% of the WT PGI activity (1264 ± 134 mU/g FW). Zymogramic analyses of PGI activity

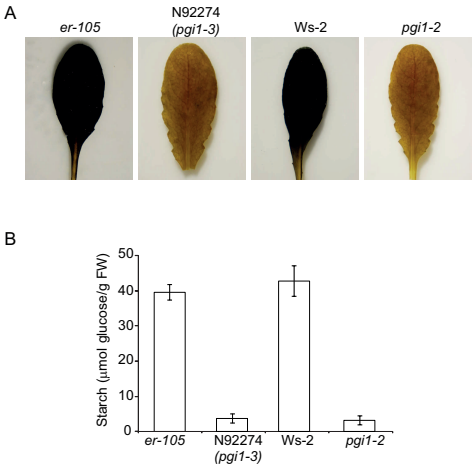

Figure 2. Leaves of the *pgi1-3* mutant accumulate low starch. (A) Iodine staining and (B) starch content of *er-105, pgi1-3,* Ws-2 and *pgi1-2* leaves. Plants were cultured on soil under LD conditions and source leaves harvested from 30 DAS plants after 12 h of illumination. In "B" values represent the mean ± SE of determinations on five independent samples.

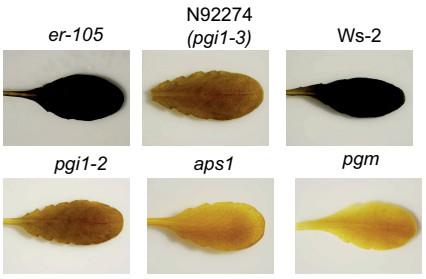

Figure 3. Iodine staining of source leaves of *er-105, pg1-3,* Ws-2, *pgi1-2, aps1* and *pgm* plants. Leaves were harvested from 30 DAS plants after 12 h of illumination. Plants were cultured under LD conditions.

revealed the occurrence of cytosolic PGI (cytPGI) and pPGI in WT leaves, but only cytPGI in *pgi1-3* leaves (**Figure 4A**). PGI activity analyses of Q-sepharose chromatography eluted fractions revealed two activity peaks in WT leaves, whereas a single peak (corresponding to cytPGI) could be detected in *pgi1-3* leaves (**Figure 4B**). The overall data thus provided strong evidence that the *pgi1-3* chloroplasts totally lack PGI activity.

Whether *pgi1-3* is a pPGI null mutation was further investigated by generating and characterizing various *pgi1-3* plants expressing the *PGI1* encoding cDNA obtained from either WT or *pgi1-3* plants under the control of the CaMV 35S promoter (designated as *pgi1-3::PGI1* and *pgi1-3::PGI1** plants, respectively). As shown in **Figure 5A**, real time RT-PCR analyses showed that leaves of plants of two independent lines each of *pgi1-3::PGI1* and *pgi1-3::PGI1** exhibited high expression levels of the transgene. Furthermore, leaves of plants of two independent *pgi1-3::PGI1* lines accumulated WT starch content whereas, similar to *pgi1-3* leaves, *pgi1-3::PGI1** leaves accumulated ca. 10-15% of the WT starch content (**Figure 5B**). The overall data thus further provided evidence that *pgi1-3* is a pPGI null allele.

Mesophyll cells of *pgi1* null mutants contain starch

We carried out TEM analyses of *pgi1-2* and *pgi1-3* mature leaves, and confocal fluorescence microscopy (CFM) analyses of mature leaves of GBSS-GFP expressing *pgi1-2* and *pgi1-3* plants. Preliminary light microscopy analyses of toluidine stained leaves showed that mesophyll cells of WT leaves produced several starch granules per chloroplast (**Figure 6A,D**). Noteworthy, these analyses also revealed that chloroplasts of *pgi1-2* and *pgi1-3* mesophyll cells contain starch granules (**Figure 6B,C,E,F**). These observations were further confirmed by TEM analyses of WT, *pgi1-2* and *pgi1-3* leaves (**Figure 6G-I**), and CFM analyses of transgenic WT, *pgi1-2* and *pgi1-3* leaves expressing the starch granule marker GBSS-GFP (**Figure 6J-L**).

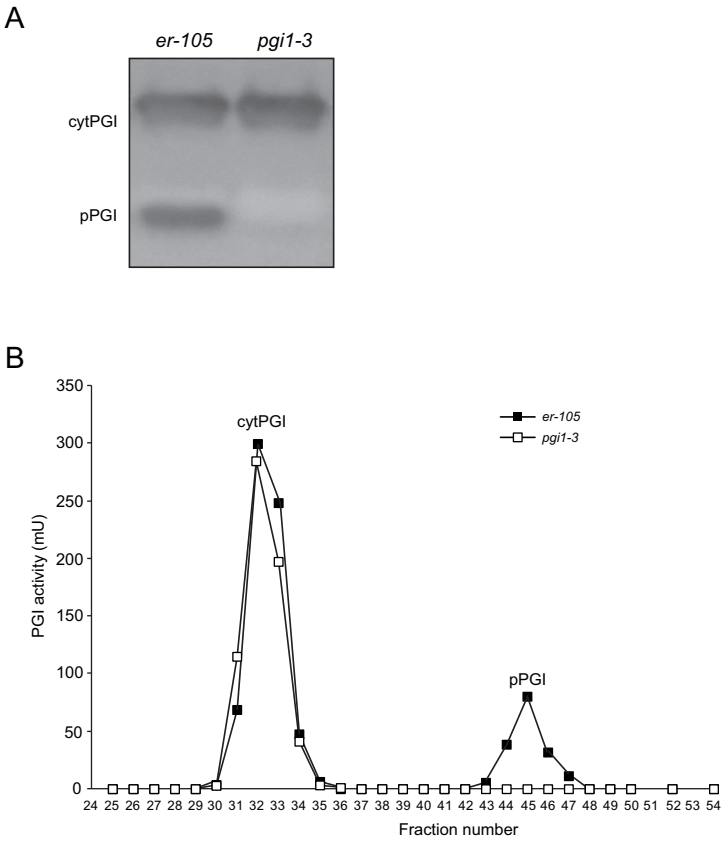

Figure 4. *pgi1-3* leaves lack pPGI activity. (A) PGI zymogram of proteins extracted from WT (*er-105*) and *pgi1-3* leaves. (B) Q-sepharose chromatography profile of PGI activity in WT and *pgi1-3* leaves. In "B", loaded WT extract contained 850 mU of total PGI activity, whereas *pgi1-3* extract loaded on the column contained 650 mU of PGI activity.

SEX1 is required for β-amylase-mediated leaf starch mobilization during the night. Mutants impaired in this function accumulate high levels of starch in the mesophyll cells (Yu et al., 2001; Edner et al, 2007). Whether mesophyll cells of *pgi1* null mutants accumulate starch was further investigated by characterizing *pgi1-2/sex1* and *pgi1-3/sex1* double mutants. The rationale behind this experimental approach was that, if *pgi1-2* and *pgi1-3* mesophyll cells do indeed accumulate starch, *pgi1-2/sex1* and *pgi1-3/sex1* leaves should accumulate more starch than *pgi1-2* and *pgi1-3* leaves, respectively. Confirming this presumption, both iodine staining (**Figure 7A**) and starch content measurement

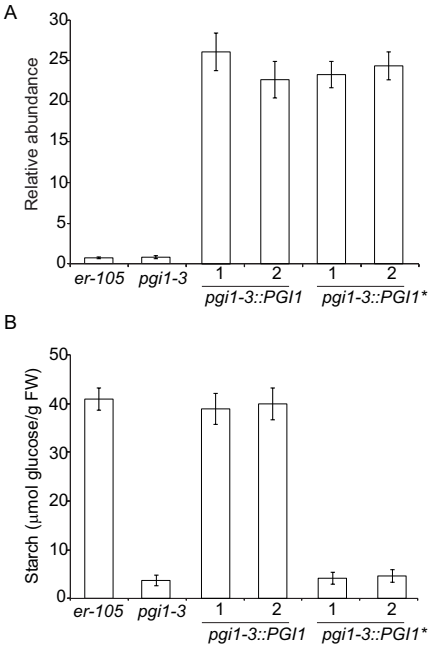

Figure 5. *pgi1-3* is a pPGI null allele. (A) RT-PCR of *PGI1* and (B) starch content in source leaves of WT (*er-105*), *pgi1-3* and two independent lines each of *pgi1-3::PGI1* and *pgi1-3::PGI1**. Plants were cultured on soil under LD conditions and leaves harvested from 30 DAS plants after 12 h of illumination. In "B" values represent the mean ± SE of determinations on five independent samples.

analyses (**Figure 7B**) revealed that *pgi1-2/sex1* and *pgi1-3/sex1* mature leaves accumulate exceedingly higher levels of starch than *pgi1-2* and *pgi1-3* leaves, respectively (see also **Figure 2**).

GPT2 is not involved in starch biosynthesis in mesophyll cells of *pgi1* leaves

Arabidopsis contains two functional plastidic G6P/Pi translocators (GPT) mainly expressed in heterotrophic tissues (GPT1 and GPT2) whose suggested role is delivery of G6P to non-green plastids as carbon skeletons for the synthesis of starch and fatty acids, or to drive the OPPP (Kang and Rawsthorne, 1996; Kammerer et al., 1998; Fox et al., 2000; Weber and Flügge, 2002; Bowsher et al., 2007; Zhang et al., 2008). Kunz et al. (2010) proposed that the occurrence of ca. 10% of the WT starch in *pgi1-2* leaves is ascribed to transport of G6P from the cytosol to the plastids of bundle sheath cells adjacent to the mesophyll and stomatal guard cells. The same authors reported that, unlike WT leaves, *pgi1-2* leaves exhibit substantial GPT activity as a consequence of the induction of *GPT2*, and proposed that GPT2 could partially contribute in the synthesis of starch in stomatal guard cells and bundle sheath cells of *pgi1-2* leaves (Kunz et al., 2010). Whether GPT2 is involved in the synthesis of starch in mesophyll cells of *pgi1* leaves was investigated by carrying out time-course analyses of the starch content in mature leaves of *pgi1* and *pgi1/gpt2* plants cultured under LD conditions. In these conditions *pgi1/gpt2* leaves accumulated as much starch as *pgi1* leaves (**Figure 8A**). We also measured the starch content in leaves of *pgi1* and *pgi1/gpt2* plants cultured under continuous light (CL) conditions, and found

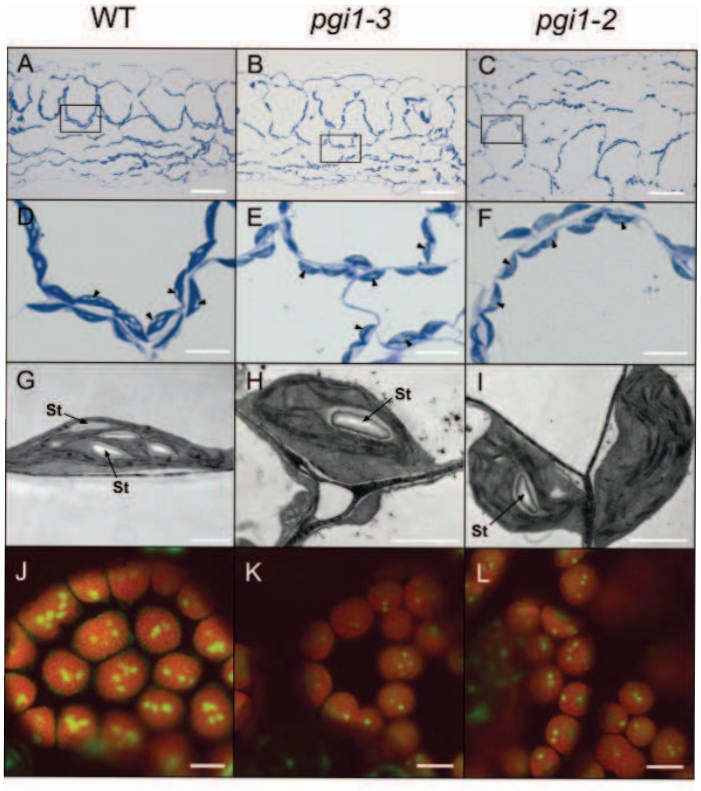

Figure 6. Microscopic analysis of starch granules in WT (*er-105*), *pgi1-2* and *pgi1-3* source leaves. (A-C) Light microscopy of toluidine stained leaf sections (Bar = 50 µm). (D-F) Magnification of mesophyll sections indicated in A-C (Bar = 10 µm). (G-I) TEM of WT, *pgi1-2* and *pgi1-3* leaves. Bar = 2 µm. (J-L) CFM of leaves of GBSS-GFP expressing WT, *pgi1-2* and *pgi1-3* leaves. Bar = 5 µm. Plants were cultured on soil under LD conditions and source leaves harvested from 30 DAS plants after 12 h of illumination. In D-F, arrows indicate the position of starch granules. St: starch.

that *pgi1* and *pgi1/gpt2* leaves accumulate comparable levels of starch (**Figure 8B**). The overall data thus show that GPT2 plays a minor role in starch biosynthesis in mesophyll cells of mature *pgi1* leaves when plants are cultured under LD and CL conditions.

Enzymatic characterization of *pgi1* leaves

To examine for possible occurrence of pleiotropic effects that could determine the starch deficient phenotype of *pgi1* leaves we measured the maximum catalytic activities of a range of enzymes closely connected to starch and sucrose metabolism in mature

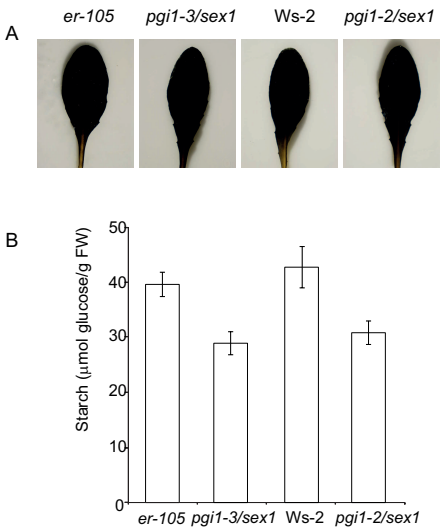

Figure 7. Introduction of *sex1* mutation into *pgi1* plants reverts the low starch content phenotype. (A) Iodine staining and (B) starch content of *er-105, pgi1-3/sex1,* Ws-2 and *pgi1-2/sex1* leaves. Plants were cultured on soil under LD conditions and leaves harvested from 30 DAS plants after 12 h of illumination. In "B" values represent the mean ± SE of determinations on five independent samples.

leaves of *pgi1-2* and *pgi1-3* plants, and in leaves of their corresponding WT plants cultured under LD conditions. Only minor changes likely due to statistical variation were observed for AGP, PPase, UGP, SPS, SuSy, acid invertase, α-amylase, adenylate kinase and Rubisco in *pgi1* leaves (**Figure 9**). However, both *pgi1-2* and *pgi1-3* leaves displayed low total PGM and soluble SS activities, and high β-amylase activity.

Increase of total β-amylase activity is a common feature of various mutants impaired in starch synthesis or breakdown (Caspar et al., 1989). Of the nine β-amylase-like proteins encoded in the Arabidopsis genome (BAM1-9), only BAM1-4 are plastidial and thus have direct access to starch (Lao et al., 1999; Sparla et al., 2006; Fulton et al., 2008). BAM1 degrades starch during the day in both mesophyll and in guard cells subjected to heat shock and osmotic stress (Kaplan and Guy., 2004; Valerio et al., 2010). BAM3 is a major determinant of leaf starch degradation during the night (Fulton et al., 2008), playing also an important role in starch degradation during the day upon cold shock (Kaplan and Guy, 2005). BAM4 is a noncatalytic protein required for starch breakdown, acting upstream of BAM1-3 (Fulton et al., 2008). Increase of total β-amylase activity in mutants impaired in starch metabolism is largely due to enhanced extraplastidial β-amylase (Caspar et al., 1989). Consistently, RT-PCR analyses revealed that the expression levels of the extraplastidial BAM5 encoding gene in *pgi1* leaves are many fold higher than those of WT leaves (**Figure 10**). Noteworthy, these analyses also revealed that the expression levels of *BAM1-3* in *pgi1* leaves are exceedingly higher than those of WT leaves, the overall data suggesting that high plastidic β-amylase activity in *pgi1* leaves can be the consequence of high expression levels of both intra- and extra-

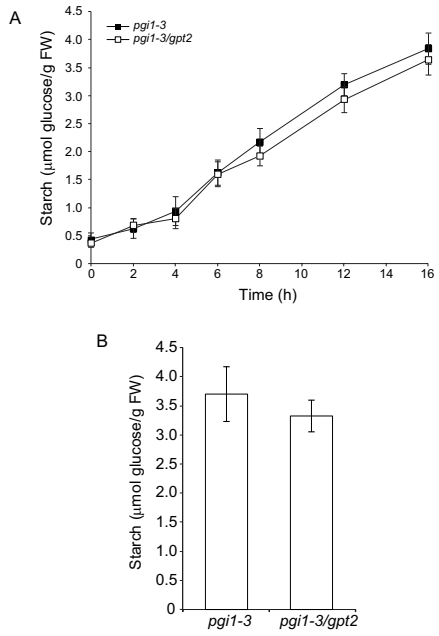

Figure 8. GPT2 is not involved in starch biosynthesis in mesophyll cells of *pgi1* leaves cultured on soil under LD and CL conditions. (A) Time-course of starch content in source leaves of *pgi1-3* and *pgi1-3/gpt2* plants cultured under LD conditions. Essentially the same results were obtained with *pgi1-2/gpt2* plants (not shown). (B) Starch content in source leaves of *pgi1-3* and *pgi1-3/gpt2* plants cultured under CL conditions. Leaves were harvested from 30 DAS plants. Values represent the mean ± SE of determinations on five independent samples. Each sample included leaves from 3 different rosettes.

plastidial β-amylases.

AGP activity is subjected to redox regulation of the small AGP subunit (APS1) (Hendriks et al., 2003; Li et al., 2012). To investigate whether the reduced levels of starch in the *pgi1* leaves could be ascribed to redox inactivation of APS1, we carried out APS1 immunoblot analyses of proteins from WT and *pgi1* leaves that had previously been extracted and electrophoretically separated under non-reducing conditions. In these conditions APS1 is present as a mixture of ca. 50 kDa active (reduced) monomers and ca. 100 kDa inactive (oxidized) dimers formed by intermolecular links involving Cys bridges. Consistent with previous reports (Hendriks et al., 2003; Li et al., 2012), these analyses revealed that most of APS1 is largely oxidized (inactive) in both WT and *pgi1* leaves (**Figure 11**). These analyses also revealed that *pgi1* leaves accumulate identical amounts of ca. 50 kDa monomers and ca. 100 kDa dimers of APS1 than WT leaves, the overall data strongly indicating that the reduced starch content of *pgi1* leaves is not ascribed to redox inactivation of APS1.

***pgi1* plants display a slow growth phenotype even under continuous light conditions**

Transitory starch is a major determinant of plant growth (Smith and Stitt, 2007). The importance of starch turnover in plant growth is demonstrated by studies of mutants that are defective in starch synthesis and mobilization. Thus, near-starchless plants impaired in AGP or pPGM show a large inhibition of growth when cultured in short day (SD)

conditions, but grow at the same rate as WT plants under CL photoperiod conditions (Caspar et al., 1985; Hanson and McHal, 1988; Lin et al., 1988; Schneider et al., 2002; Ragel et al., 2013). Previous studies on *pgi1* mutants did not include analyses of plant

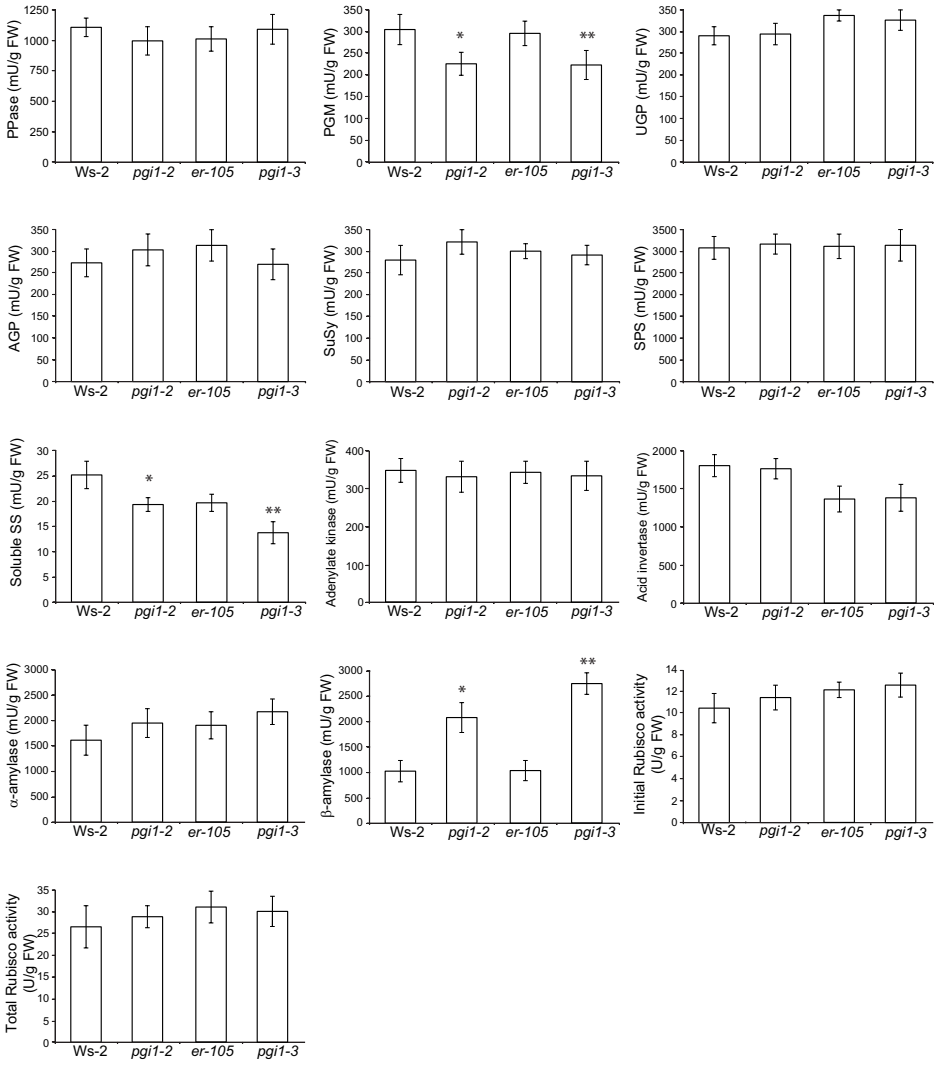

Figure 9. Activities of enzymes closely connected to starch and sucrose metabolism in source leaves of WT (*er-105* and Ws-2), *pgi1-2* and *pgi1-3* plants. Plants were cultured on soil under LD conditions. Fully developed, source leaves were harvested from 30 DAS plants after 12 h of illumination. Values represent the mean ± SE of determinations on four independent samples. Asterisks indicate significant differences based on Student´s t-tests. (*P<0.05, *pgi1-2* vs. Ws-2; **P<0.05, *pgi1-3* vs. *er-105*).

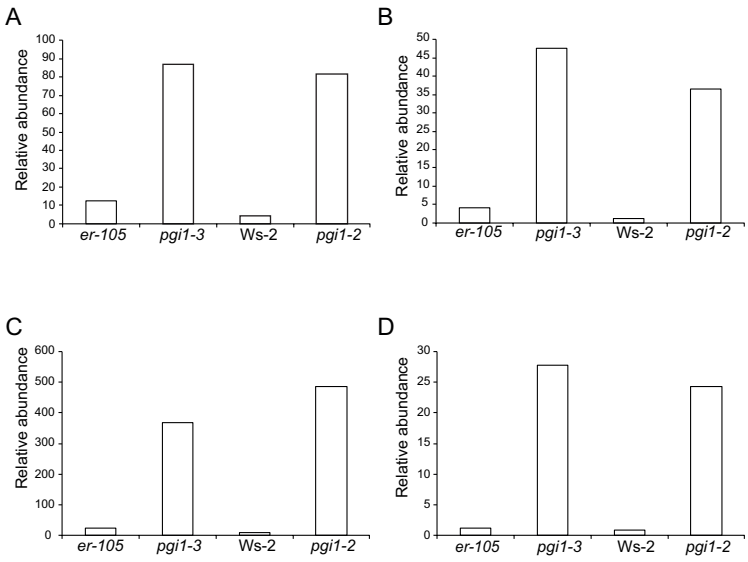

Figure 10. High expression levels of plastidial and extraplastidial β-amylase encoding genes in *pgi1* plants. RT-PCR of (A) *BAM1,* (B) *BAM2,* (C) *BAM3* and (D) *BAM5* in source leaves of WT (*er-105* and Ws-2), *pgi1-2* and *pgi1-3* plants. Fully developed, source leaves were harvested from 30 DAS plants after 16 h of illumination.

growth under CL conditions (Yu et al., 2000; Kunz et al., 2010, 2014). We thus carried out time-course analyses of FW of rosettes of *pgi1-2, pgi1-3* and WT plants cultured either under SD (12 h light/12 h dark) or CL photoperiod conditions. We also carried out analyses of growth of near-starchless *aps1* and *pgm* mutants (Columbia (Col-*O*) background)) impaired in AGP and pPGM, respectively. As shown in **Figure 12**, *aps1* and *pgm* plants displayed a marked slow growth phenotype when cultured under SD conditions, and grew as WT plants when cultured under CL conditions. In clear contrast, *pgi1-2* and *pgi1-3* displayed a slow growth phenotype at

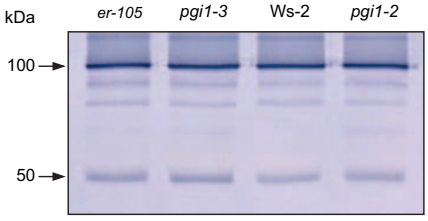

Figure 11. Non-reducing western blot of APS1 in leaves of *er-105, pg1-3,* Ws-2 and *pgi1-2* plants. Leaves were harvested from 30 DAS plants after 12 h of illumination. Plants were cultured under LD conditions.

any photoperiod regime (**Figure 12**) strongly indicating that (a) reduced starch turnover is not the reason of the slow growth phenotype of *pgi1* mutants, and (b) pPGI is an important determinant of plant growth.

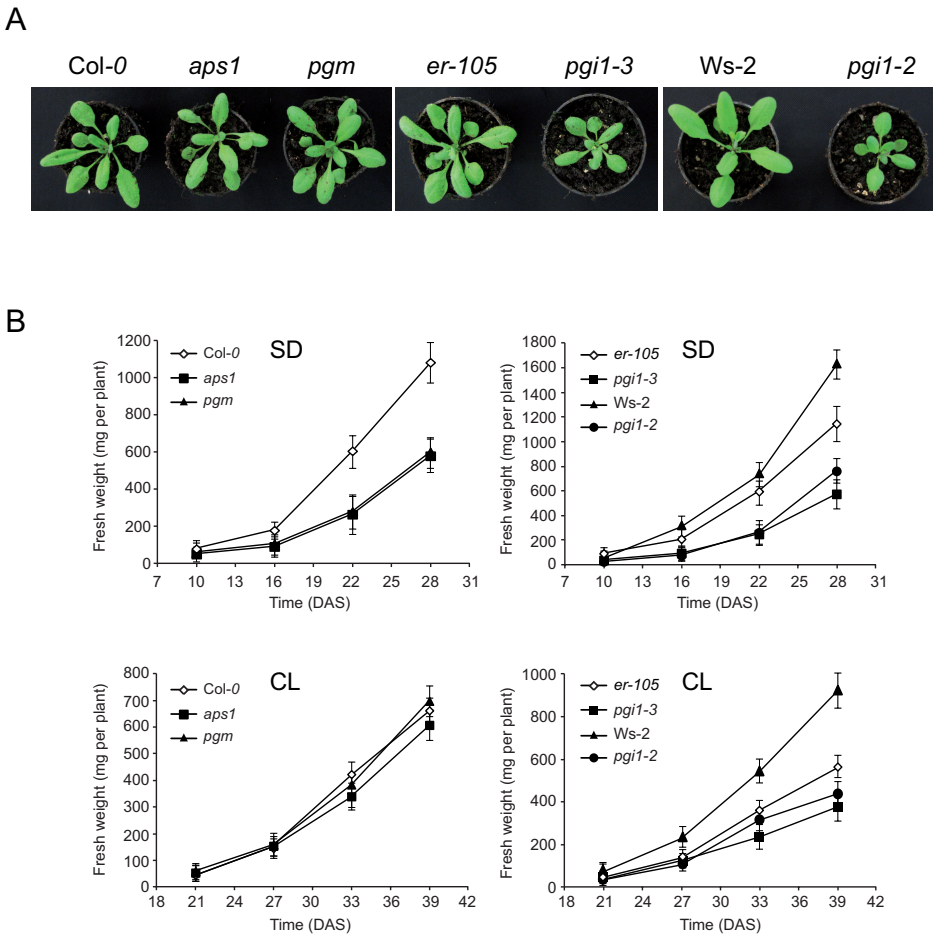

Figure 12. *pgi1-2* and *pgi1-3* leaves display a slow growth phenotype under SD and CL photoperiod conditions. (A) Photographs of 25 DAS WT, *aps1, pgm, pgi1-2* and *pgi1-3* plants cultivated in growth cabinets under CL conditions. (B) Time-course of FW of rosettes of WT, *aps1, pgm, pgi1-2* and *pgi1-3* plants cultured on soil under SD and CL conditions. In "B", values represent the mean ± SE of determinations on four independent samples.

***pgi1* leaves have reduced photosynthetic capacity even under continuous light conditions**

The low rates of growth and leaf starch accumulation of *pgi1* plants in both SD and CL conditions (see above) pointed to the possible occurrence of reduced photosynthetic capacity of *pgi1* leaves and/or altered mitochondrial respiration. We thus measured A_n in mature leaves of *pgi1-2* and *pgi1-3* plants under saturating light intensity of 350 µmol m^{-2} s^{-1} and with a CO_2 concentration of 450 µmol mol^{-1}, and compared with those of WT plants when cultured under either LD or CL photoperiod conditions. We also analyzed the g_s under the same conditions. Moreover, we evaluated respiration rates in darkened *pgi1-2* and *pgi1-3* leaves. These analyses revealed that whereas respiration rates of darkened *pgi1* leaves were comparable to that of WT leaves (not shown), the photosynthetic capacities of *pgi1-2* and *pgi1-3* mature leaves were ca. 40-50% lower than that of WT leaves at any photoperiod regime. Furthermore, g_s values in *pgi1* plants were moderately (although not significantly) lower than those of WT plants (**Figure 13**).

A strong reduction in A_n in *pgi1* plants without a parallel strong reduction in g_s (and thus limitation in CO_2 availability) would suggest the occurrence of biochemical limitations restricting photosynthesis. To test this hypothesis, we measured A_n in *pgi1* plants cultured under both LD and CL conditions, and under varying *Ci*. We also measured the ETR with respect to varying *Ci*. As shown in **Figure 14**, irrespective of the photoperiod conditions, *pgi1* plants had strongly reduced values of A_n when compared with WT plants. Analyses of the *Vcmax*, the TPU and the *Jmax* (that is equivalent to the ribulose-1,5-bisP (RuBP) regeneration rate) calculated from the A_n/Ci curves revealed that *Vcmax* of Rubisco in *pgi1* leaves is significantly lower than that of WT leaves (**Table 3**). This indicated that the reduction in A_n observed in *pgi1* plants was related to differences in *in planta* Rubisco activity. Furthermore, *pgi1* plants displayed significant reductions in *Jmax* and TPU with respect to WT plants, thus indicating a role of pPGI in the protection of the electron transport leading to the regeneration of RuBP and the capacity of the chloroplast reactions to use triose-phosphates. The absence of significant differences on *Jmax/Vcmax* between *pgi1* leaves and WT leaves indicate that reductions in *Jmax* and *Vcmax* in *pgi1* leaves are the consequence of similar factor(s), most likely perturbation in photosynthetic energy transduction in *pgi1* plants. Supporting this view, ETR values in WT plants were exceedingly higher than those of *pgi1* plants under any *Ci* conditions (**Figure 14**).

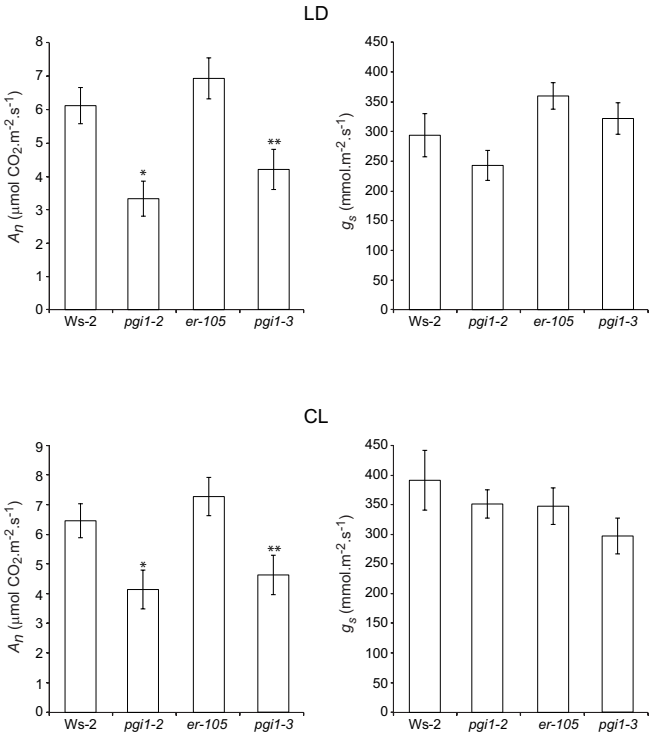

Figure 13. *pgi1* leaves have reduced photosynthetic capacity. The graphics represent net CO_2 uptake (A) and stomatal conductance (g_s) in source leaves of WT (Ws-2 and *er-105*), *pgi1-2* and *pgi1-3* plants cultured on soil under SD and CL conditions. Values represent the mean ± SE of determinations on four independent samples. Asterisks indicate significant differences based on Student´s t-tests. (*P<0.05, *pgi1-2* vs. Ws-2; **P<0.05, *pgi1-3* vs. *er-105*).

Metabolic characterization of *pgi1* leaves

We measured metabolites content in leaves of *pgi1-2, pgi1-3* and their corresponding WT plants cultured under LD conditions. Under these conditions *pgi1-2* and *pgi1-3* leaves accumulated nearly WT levels of glucose, fructose, sucrose, G6P, F6P and G1P (**Figure 15**). Levels of ATP in *pgi1* leaves were slightly (but not significantly) lower than those of WT leaves, whereas levels of ADP and AMP in *pgi1-2* and *pgi1-3* leaves were significantly higher than in their corresponding WT leaves. Consistently, adenylate energy charge of *pgi1-2* and *pgi1-3* leaves was lower than that of WT leaves (**Figure 15**). The NADPH/NADP and NADH/NAD ratios in *pgi1* leaves were significantly lower than in WT leaves (**Figure 15**), which would indicate that pPGI is a determinant

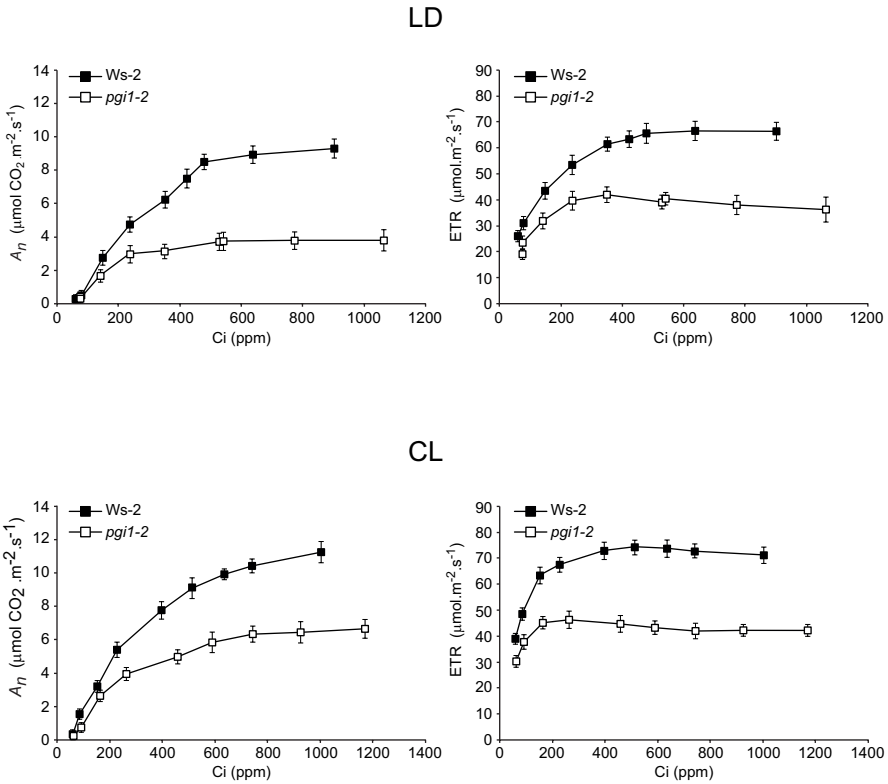

Figure 14. *pgi1-2* leaves have reduced photosynthetic capacity and ETR. (A) CO_2 assimilation rates, and (B) photosynthetic electron transport at different intercellular CO_2 concentrations in WT and *pgi1-2* source leaves. Plants were cultured on soil under LD conditions. Values represent the mean ± SE (n=5)

of the cellular redox potential. Consistent with previous reports showing the occurrence of important ADPG sources other than the pPGI-pPGM-AGP pathway (Bahaji et al., 2011, 2014a), *pgi1-2* and *pgi1-3* leaves accumulated WT ADPG content (**Figure 15**).

pgi1 leaves accumulate low levels of active forms of cytokinins derived from the plastidic MEP pathway

pPGI is involved in the OPPP and glycolytic pathways in non-illuminated leaves and heterotrophic organs. Glyceraldehyde 3-phosphate (GAP) is a glycolytic and OPPP metabolic intermediate that acts as substrate for the initial reaction of the plastidic MEP

pathway involved in the synthesis of isoprenoids (Lichtenthaler et al., 1997; Phillips et al., 2008; Pulido et al., 2012) (**Figure 16**). Therefore, pPGI could potentially act as a

Table 3. Photosynthetic parameters of WT (Ws-2 and *er-105*), *pgi1-2* and *pgi1-3* source leaves. Plants were cultured under LD and CL conditions. Values represent the mean ± SE of determinations on five independent samples.

Line	Jmax	Vcmax	TPU
Ws-2 (LD)	77.5 ± 6.6	56.0 ± 4.0	2.3 ± 0.29
pgi1-2 (LD)	24.3 ± 1.2	21.6 ± 1.9	1.4 ± 0.20
Ws-2 (CL)	65.5 ± 3.4	29.4 ± 2.9	2.6 ± 0.21
pgi1-2 (CL)	30.2 ± 1.9	17.7 ± 3.1	1.9 ± 0.15
er-105 (LD)	63.1 ± 6.5	38.9 ± 4.0	2.4 ± 0.22
pgi1-3 (LD)	30.8 ± 6.5	15.1 ± 1.4	1.5 ± 0.12
er-105 (CL)	59.3 ± 4.4	64.5 ± 4.3	2.6 ± 0.30
pgi1-3 (CL)	38.1 ± 2.7	36.7 ± 4.2	1.7 ± 0.19

determinant for the synthesis of plastidic MEP-pathway derived isoprenoid compounds. Among different plastidic isoprenoid derived molecules, CKs have been shown to act as major determinants of growth, energy status, starch content and photosynthesis in mature leaves (Synková et al., 1999; Yang et al., 2003; Yaronskaya et al., 2006; Werner et al., 2008; Cortleven et al., 2011; Cortleven and Valcke, 2012; Erickson et al., 2014). Therefore, we considered of interest to investigate the possible involvement of pPGI in CKs metabolism by measuring the levels of different CKs in mature leaves of both Ws-2 and *pgi1-2* plants. These analyses revealed that the lack of pPGI causes a decrease of the total content of plastidic-type, MEP pathway-derived isopentenyladenine (iP)- and tZ-type CKs, mainly as a consequence of the reduction of the main precursors of the active CKs iPRMP and tZRMP (**Table 4, Figure 16**). The tZRMP content in *pgi1-2* leaves was only 32% of that of WT leaves (**Table 4**). As a consequence, the levels of tZ (the most abundant biologically active CKs) and its riboside tZR in *pgi1-2* leaves were only 51% and 28% of those of WT leaves, respectively (**Table 4**). The iPRMP content in *pgi1-2* leaves was ca. 70% of that of WT leaves, whereas the iPR content in *pgi1-2* leaves was ca. 15% of that of WT leaves (**Table 4**), pointing to the possible occurrence of a general

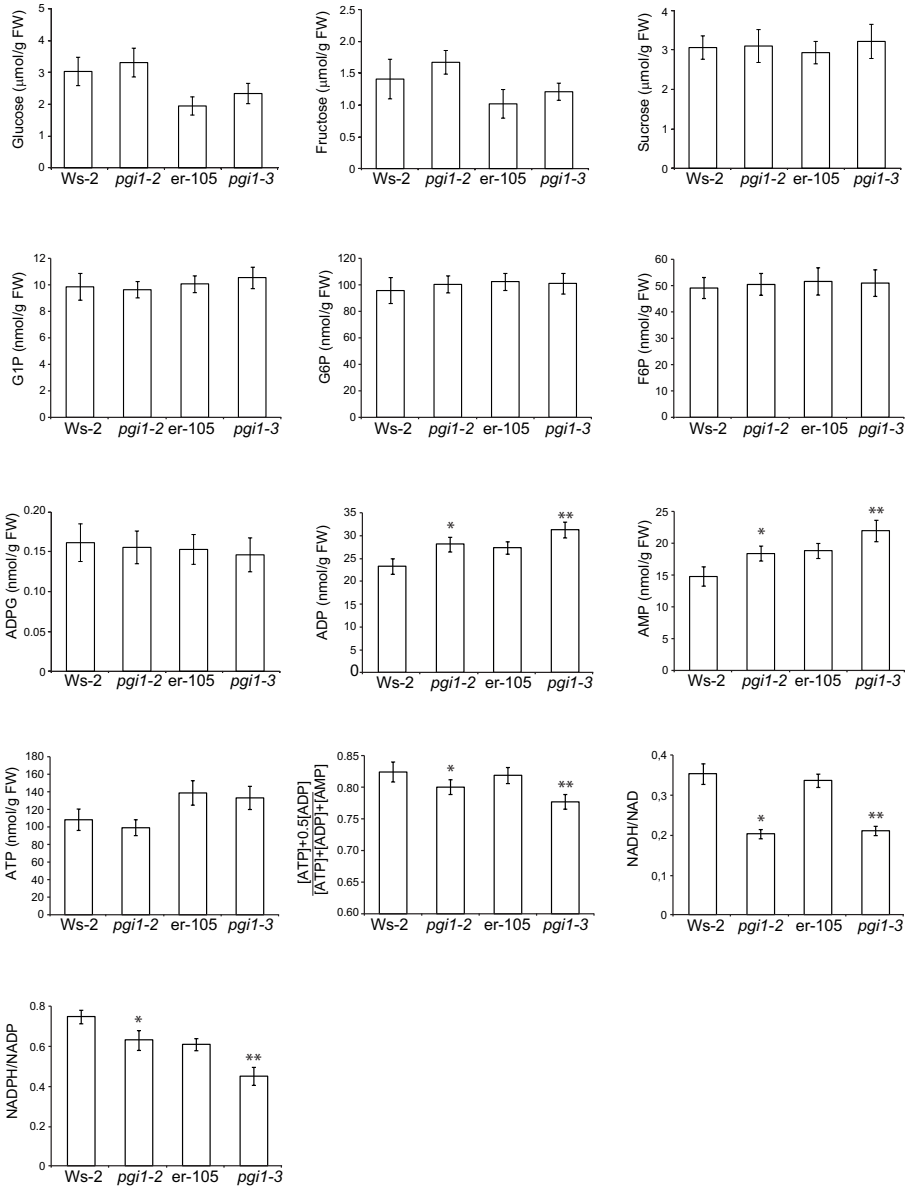

Figure 15. Metabolites content in mature leaves of WT (Ws-2 and *er-105*), *pgi1-2* and *pgi1-3* plants cultured on soil under LD conditions. Fully developed, source leaves were harvested from 30 DAS plants after 12 h of illumination. Values represent the mean ± SE of determinations on five independent samples. Each sample included leaves from 3 different rosettes. Asterisks indicate significant differences based on Student´s t-tests. (*P<0.05, *pgi1-2* vs. Ws-2; **P<0.05, *pgi1-3* vs. *er-105*).

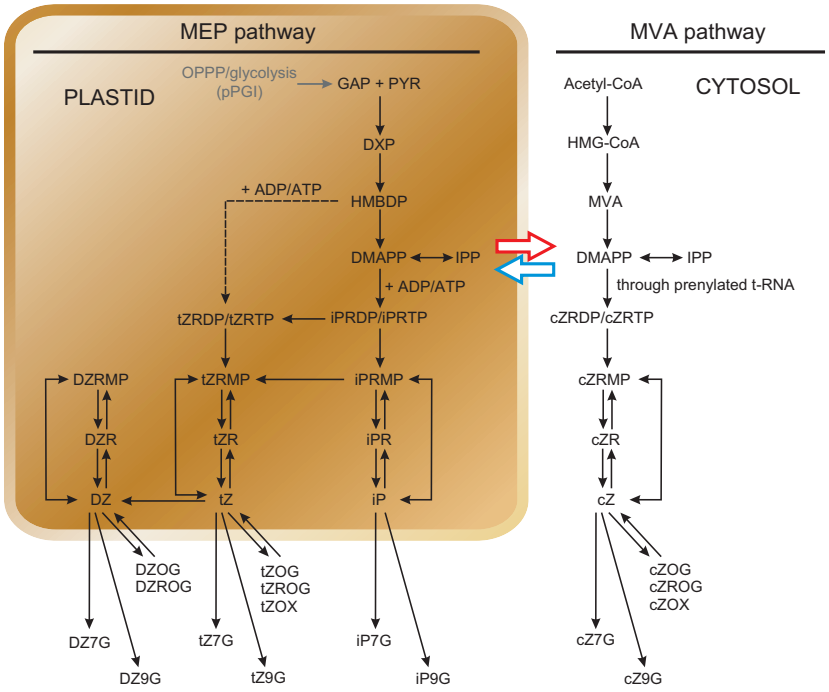

Figure 16. Scheme of CK biosynthesis through plastidic MEP- and cytosolic MVA-pathway. Black arrows show the biosynthesis, interconversions and metabolism flow of CKs in Arabidopsis cell (adapted from Spíchal 2012) The dashed arrow indicates the iPRMP-independent pathway of tZ biosynthesis (Åstot et al., 2000). The blue and the red arrows indicate a hypothetical exchange of common precursor(s) between the MEP and MVA pathways (adapted from Kasahara et al., 2004). GAP, glyceraldehyde 3-phosphate; PYR, pyruvate; DXP, 1-deoxy-D-xylulose 5-phosphate

down-regulation of conversion of active CK free bases to their corresponding ribosides in *pgi1-2* plants. The content of the biologically less active DHZ in *pgi1-2* leaves was 50% of that of WT leaves (**Table 4**). The levels of the irreversibly glycosylated N9- and N7-glycosylated CKs (tZ9G, tZ7G, iP9G) and the reversibly *O*-glycosylated forms of tZ (tZOG and tZROG) were significantly lower than those of WT leaves (**Table 4**). Noteworthy, virtually no differences were found between the content of cytosolic mevalonate (MVA) pathway derived *cis*-zeatins (cZ) in *pgi1-2* and WT leaves (**Table 4**). The overall data would indicate that the lack of pPGI causes reduction of plastidic MEP pathway derived CKs, but not of cytosolic MVA pathway derived CKs in *pgi1-2* leaves.

Table 4. CKs content (pmol g^{-1} DW) in leaves of 30 DAS Ws-2 and *pgi1-2* plants. CK precursors, transport forms, active forms and glycosylated inactive forms are separated in two groups based on their origin from MEP and MVA pathway, respectively. Total sums and corresponding percentage is shown for individual forms. Symbols indicate significant differences according to ANOVA. *< 0.05; **<0.01; **<0.001; ns, not significant; n.a., not analyzed.

		MEP pathway (plastid) derived CKs			MVA pathway (cytosol) derived CKs		
		WS-2	*pgi1-2*		WS-2	*pgi1-2*
Precursors	iPRMP	465 ± 43.4	329 ± 34.5 ns	cZMRP	51.4 ± 8.5	48.2 ± 7.4 ns
	tZRMP	**845.6** ± **100.4**	**269.1** ± **20.5** **			
	DHZMP	4.8 ± 0.3	3.5 ± 0.2 ns			
	∑ (%)	1315.4 (100%)	601.6 (45.7%)		(100%)	93.8%
Transport forms	iPR	**34.8 ± 4.4**	**5.5 ± 0.5** ***	cZR	2.9 ± 0.2	2.0 ± 0.2 ns
	tZR	**100.2 ± 10.5**	**28.4 ± 4.6** **			
	DHZR	**1.6 ± 0.2**	**2.1 ± 0.1** *			
	∑ (%)	136.6 (100%)	36 (26.4%)		(100%)	69%
Active forms	iP	9.7 ± 0.8	11.8 ± 1.1 ns	cZ	1.8 ± 0.2	1.6 ± 0.1 ns
	tZ	**45.3 ± 4.2**	**23.3 ± 1.5** **			
	DHZ	**0.8 ± 0.18**	**0.4 ± 0.01** **			
	∑ (%)	55.8 (100%)	35.5 (63.6%)		(100%)	88.9%
Glycosylated (inactive) forms	iP7G	10.2 ± 0.7	12.7 ± 1.2 ns	cZ7G	n.a.	n.a.
	tZ7G	**65.9 ± 2.7**	**42.5 ± 2.1** *			
	DHZ7G	6.1 ± 0.6	5.8 ± 0.1 ns			
	iP9G	**16.7 ± 2.0**	**7.9 ± 0.2** ***	cZ9G	**0.9 ± 0.1**	**0.5 ± 0.0** **
	tZ9G	**445.4 ± 8.0**	**216.7 ± 12.9** **			
	DHZ9G	4.2 ± 0.6	3.5 ± 0.2 ns			
	tZOG	193.4 ± 5.1	146.3 ± 8.9 ns	cZOG	**19.0 ± 2.1**	**32.1 ± 0.9** **
	DHZOG	**1.8 ± 0.1**	**3.9 ± 0.3** **			
	tZROG	18.5 ± 0.5	9.7 ± 1.3 ns	cZROG	23.0 ± 3.0	24.0 ± 2.5 ns
	DHZROG	1.2 ± 0.1	1.3 ± 0.2 ns			
	∑ (%)	763.4 (100%)	450.3 (59%)		42.9 (100%)	56.6 (131.9%)
Total ∑ (%)		2271.2 (100%)	1123.4 (49.5%)		47.6 (100%)	60.2 (126.5%)

Exogenous application of CKs reverts the starch deficient phenotype of *pgi1* plants

pgi1 mutants exhibit symptoms indicative of reduced plastidic CKs content such as reduced size, low starch content and reduced photosynthetic capacity at any photoperiod condition (see above). Whether the low starch content phenotype of *pgi1* leaves could be the consequence, at least in part, of reduced plastidic CKs (see **Table 4**) was investigated by measuring the starch content in leaves of adult *pgi1* plants cultured for 2 days in solid MS medium supplemented with different concentrations of tZ. As shown in **Figure 17**, these analyses revealed that exogenous CK application largely reverts the starch deficient phenotype of *pgi1* leaves.

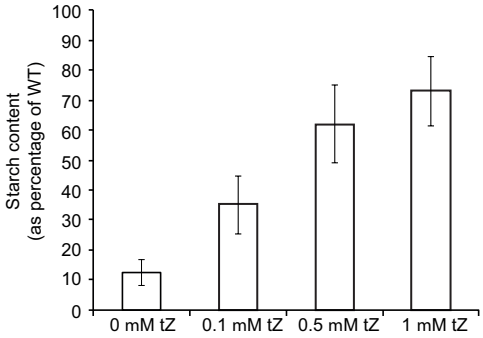

Figure 17. Starch content (as percentage of starch occurring in WT leaves) in leaves of *pgi1-2* cultured in MS medium including the indicated concentrations of tZ. Values represent the mean ± SE of determinations on four independent samples.

DISCUSSION

The initial objective of this work was to characterize the *pgi1-3* mutant both at the molecular and biochemical levels, using *pgi1-2* as reference. Contrary to expectations, during the course of our studies we found that mesophyll cells of mature leaves of the two *pgi1* null mutants accumulate ca. 10-15% of the WT starch content (**Figure 2, Figure 5, Figure 6**). The discrepancy between our results showing the presence of starch granules in mesophyll cells of *pgi1* leaves and those of Kunz et al. (2010) showing that starch granules are restricted to bundle sheath cells adjacent to the mesophyll and stomatal guard cells are likely ascribed to the use of different growth conditions. Thus, whereas Kunz et al. (2010) carried out their experiments using plants cultured under SD conditions, we cultured the plants under LD and CL conditions. Another possible reason explaining the discrepancy between our results and those of Kunz et al. (2010) is the use of different microscopic techniques.

The occurrence of starch granules in the chloroplasts of mesophyll cells conflicts with the widely accepted view that the whole photosynthesis-driven starch biosynthetic process in mesophyll cells solely occurs in the chloroplast by means of the Calvin-Benson cycle-pPGI-pPGM-AGP-SS pathway. Therefore, it is conceivable that at least 10-15% of the starch accumulated in the leaf mesophyll cells is produced by metabolic pathway(s) wherein (a) the Calvin-Benson cycle is not directly connected to the starch biosynthetic pPGM-AGP-SS pathway by means of pPGI, and (b) carbon units linked

to starch biosynthesis are imported from the cytosol. Taking into account that (a) the photosynthetic capacity of *pgi1* leaves at any photoregime is exceedingly lower than that of WT plants (**Figure 13, Figure 14**), and (b) the adenylate energy charge and cellular redox potential of *pgi1* leaves are lower than those of WT leaves (**Figure 15**), it is highly likely that starch deficiency of *pgi1* leaves is partially the consequence of either reduced CO_2 fixation capacity and/or low energy status and cellular redox potential, and not only the consequence of lack of pPGI-mediated flow between the Calvin-Benson cycle and the pPGM-AGP-SS starch biosynthetic pathway.

Chloroplasts of mature leaves are not capable of transporting G6P (Quick et al., 1995). Consistently, studies of functional reconstitution of membrane proteins in proteoliposomes revealed that chloroplasts from Ws-2 and *er-105* plants do not transport G6P (cf. Table 2 in (Kunz et al., 2010)). These studies also revealed that 97% of capacity of *pgi1-2* chloroplasts to transport G6P depends on GPT2, since G6P/Pi transport activity in proteoliposomes prepared from *pgi1-2/gpt2* leaves was only 3% of that found in proteoliposomes prepared from *pgi1-2* leaves (cf. Table 2 in (Kunz et al., 2010)). Although this would indicate in principle that GPT2 could be involved in the incorporation of cytosolic G6P for its subsequent conversion into starch in *pgi1-2* mature leaves, results presented in **Figure 8** showing that the rate of starch accumulation in mature leaves of *pgi1* plants cultured under LD and CL conditions is comparable to that of leaves of *pgi1/gpt2* plants provide strong evidence that GPT2-mediated incorporation of cytosolic G6P into chloroplasts plays a minor role in the synthesis of starch in mesophyll cells of *pgi1* leaves. Although GPT1 only catalyzes the 3% of the total GP6/Pi transport activity in *pgi1-2* leaves, a possibility cannot be ruled out that GPT1 may contribute to some extent to the production of starch in mesophyll cells of *pgi1* plants cultured under LD and CL conditions.

Among a large group of plastidic metabolite transporters functionally related to the metabolism of sugars or sugar derivatives, none of them is able to transport G1P (Kammerer et al., 1998; Eicks et al., 2002). However, Fettke et al. (2011) reported that the envelope membranes of chloroplasts of mesophyll cells possess a yet to be identified G1P transport machinery enabling the incorporation into the stroma of cytosolic G1P. According to these authors, however, such mechanism would only account for the accumulation of 1% of the WT starch, explaining the occurrence of trace amounts of starch in mutants impaired in pPGM. Thus, incorporation of cytosolic G1P into the chloroplast

and its subsequent conversion into starch could minimally explain the accumulation of some starch in *pgi1* mesophyll cells chloroplasts. Chloroplasts possess a glucose transporter (pGlcT) (Weber et al., 2000) and hexokinase (Giese et al., 2005) potentially enabling the incorporation of cytosolic glucose and subsequent conversion into G6P thus bypassing the pPGI step in *pgi1* leaves. However *pglct* mutants accumulate WT levels of starch during the day (Cho et al., 2011), and GlcT has been shown to act in the export to the cytosol of glucose from the starch breakdown during the night rather than in the import of cytosolic glucose to the chloroplast (Weber et al., 2000; Cho et al., 2011). Chloroplasts from mature leaves also possess a yet to be identified ADPG transport machinery (Pozueta-Romero et al., 1991). Taking into account that a sizable pool of ADPG linked to starch biosynthesis has a cytosolic localization in leaves (Baroja-Fernández et al., 2004; Bahaji et al., 2011, 2014a) it is likely that starch biosynthesis in mesophyll cells of *pgi1* mature leaves involves the cytosolic production of ADPG and its subsequent transport into the chloroplast and conversion into starch. Needless to say, further research will be necessary to identify the cytosolic hexose molecules entering the chloroplast for their subsequent metabolization into starch in *pgi1* leaves.

The occurrence of a starch biosynthetic pathway in mesophyll cells involving the incorporation into the chloroplast of cytosolic hexoses (either glucose, hexose-phosphates and/or ADPG) likely provides a clue to explain still enigmatic results reported almost 60 years ago using green leaves exposed to $^{14}CO_2$ for a short period of time (Kandler and Gibbs, 1956; Gibbs and Kandler, 1957). According to the widely accepted view of starch biosynthesis, leaves exposed to $^{14}CO_2$ for a short period of time should produce starch with ^{14}C symmetrically distributed in the glucose molecules. However, leaves shortly exposed to $^{14}CO_2$ synthesized starch with ^{14}C asymmetrically distributed in the glucose molecules (Kandler and Gibbs, 1956; Gibbs and Kandler, 1957). Noteworthy, the same asymmetric distribution of ^{14}C was found in the glucose moiety of hexose-phosphates, sucrose and nucleotide-sugars (Kandler and Gibbs, 1956; Gibbs and Kandler, 1957). According to the models of starch biosynthesis involving the incorporation into the chloroplast of cytosolic hexoses (reviewed in Bahaji et al., 2014b), triose-Ps exported from the chloroplast to the cytosol can be channeled into the OPPP, thereby leading to a randomization of the carbons that gives rise to the asymmetric ^{14}C distribution observed in sucrose, hexose-phosphates and nucleotide-sugars (see Figure 4 in ref. Bahaji et al., 2014b). Asymmetrically labeled cytosolic hexoses would enter the

chloroplast, thus explaining why leaves shortly exposed to $^{14}CO_2$ synthesize starch and sucrose with identical ^{14}C asymmetric distribution. Also, the occurrence of metabolic pathways involving both the incorporation into the chloroplast of cytosolic hexoses, and the occurrence of simultaneous synthesis and breakdown of starch in the illuminated chloroplast (see Bahaji et al., 2014b and references contained therein) likely provide clues to explain the rapid formation of radiolabelled maltose in leaves when plants are cultured in $^{13}CO_2$ or $^{14}CO_2$-enriched environments (Linden et al., 1975; Szecowka et al., 2013), and the asymmetric labeling of maltose formed by illuminated leaves cultured for a short period of time in $^{14}CO_2$-enriched environment (Linden et al., 1975).

It is widely accepted that starch turnover is a major determinant of plant growth as demonstrated by studies of mutants that are defective in starch synthesis and mobilization. Some authors postulated that restricted growth of starch-deficient plants is the consequence of carbon starvation occurring every night due to the inability to accumulate starch during the day or to degrade it during the night (Schulze et al., 1991; Huber and Hanson, 1992; Smith and Stitt, 2007). Others postulated that acute deficiency of sugars occurring in starch mutants during the end part of the dark period temporary inhibits growth (Gibon et al., 2004). Restricted growth of starch-deficient plants has also been ascribed to regulatory imbalances in photosynthetic capacities and enzymatic activities triggered by high sugars during the day (Caspar et al., 1985; Stitt, 1991; Koch, 1996). However, as shown in **Figure 15**, *pgi1* leaves accumulate nearly WT levels of soluble sugars. Thus, factors other than high sugar levels must be responsible for the restricted growth (**Figure 12**), reduced photosynthetic capacity (**Figure 13, Figure 14**) and altered activities of some carbohydrate metabolism enzymes (**Figures 9**) of *pgi1* mutants.

Results presented in **Figure 12** showing that *pgi1* plants cultured under CL conditions are smaller than WT plants, whereas the near-starchless *aps1* and *pgm* plants display a WT growth phenotype in the same culture conditions, provide strong evidence that (a) reduced starch turnover is not the reason of the slow growth phenotype of *pgi1* mutants, and (b) pPGI is an important determinant of plant growth. pPGI is involved in the regeneration of G6P molecules in the OPPP in heterotrophic organs and non-illuminated leaves. OPPP provides precursors for the synthesis of RNA, DNA and phenolic compounds such as aromatic amino acids, lignin, flavonoids and phytoalexins (Herrman, 1995). This metabolic pathway also provides NADPH necessary for biosynthetic redox reactions involved in lipid biosynthesis and nitrogen assimilation (Kang and Raw-

sthorne, 1996; Bowsher et al., 2002; Bowsher et al., 2007; Jong et al., 2014), and for NADP-thioredoxin reductase (NTRC) dependent processes such as supply of reductant necessary for detoxifying hydrogen peroxide in the dark, and maintaining the redox homeostasis of plastids which in turn determines plant growth and development (Pérez-Ruiz et al., 2006; Lepistö et al., 2009; Kirchsteiger et al., 2012). Noteworthy, it has been reported that G6P metabolization within the OPPP is required for generating a signal that governs the regulation of root mediated acquisition of nitrogen and sulfur necessary for amino acid synthesis (Lejay et al., 2008). Also, previous reports have shown that, similar to plants impaired in pPGI, mutants impaired in other plastidic OPPP enzymes such as 6-phosphogluconolactonase display a reduced size phenotype (Xiong et al., 2009; Bussell et al., 2013). Therefore, it is conceivable that the reduced size of *pgi1* mutants is ascribed, at least in part, to impairments in some OPPP-dependent processes occurring in heterotrophic organs that are important for growth.

Results presented in **Figures 13 and 14** show that the photosynthetic capacities of starch deficient *pgi1* plants are lower than that of WT plants at any photoregime. Noteworthy, recent studies have shown that the near-starchless *pgm1* mutant impaired in pPGM has WT photosynthetic CO_2 fixation rates (Brauner et al., 2014; our unpublished results), providing evidence that starch turnover exerts a minor influence on the photosynthetic capacity of the plant. This would strongly indicate that (a) the lack of pPGI, but not the reduced levels of starch, is the reason for the reduced photosynthetic capacity of *pgi1* plants, and (b) pPGI is an important determinant of the photosynthetic capacity of the plant. That *pgi1* mutants display reduced *Vcmax*, TPU and *Jmax* (**Table 3**) and ETR (**Figure 14**) at any photoregime strongly indicates that impaired Rubisco carboxylation activity, together with limitations in RubP regeneration (as a consequence of reduced electron flux towards Rubisco carboxylation) and reduced capacity to use triose-phosphates are responsible for the low photosynthetic capacity of these mutants.

GAP and pyruvate are the substrates for the initial reaction of the plastidic MEP pathway involved in the synthesis of isoprenoid derived molecules such as CKs (**Figure 16**) (Pulido et al., 2012). In non-illuminated leaves and heterotrophic organs GAP can be produced in the OPPP and glycolytic pathways involving pPGI. On the contrary, pyruvate biosynthesis in heterotrophic plastids largely depends on pPGI independent pathways, since enzymatic activities of the lower part of the glycolytic pathway such as phosphoglycerate mutase are marginally low in heterotrophic plastids (Frehner et al.,

1990; apRees et al., 1975; Journet and Douce, 1985). Plastidic MEP pathway derived CKs are mainly synthesized in roots and transported to the aerial parts of the plant, where they regulate plant growth (Ko et al., 2014). Noteworthy, in addition to their involvement in regulating plant growth and development, CKs act as major determinants of photosynthetic activity, Jmax and TPU by regulating the biogenesis of chloroplasts, and providing components of the electron transport chain, structural proteins and the enzymes for their formation (Synková et al., 1999; Yang et al., 2003; Yaronskaya et al., 2006; Rivero et al., 2009; Cortleven et al., 2011; Cortleven and Valcke, 2012). Also, CKs maintain stomata open (Tanaka et al., 2006; Havlová et al., 2008). Moreover, CKs exert a positive effect on starch accumulation both in leaves and heterotrophic sink organs (Werner et al., 2008; Peleg et al., 2011; Erickson et al., 2014), most likely by regulating the expression of starch metabolism related genes (Miyazawa et al., 1999). Importantly, results presented in **Table 4** showed that total levels of plastidic MEP pathway derived forms of CKs in *pgi1-2* leaves are low when compared with WT leaves, which provides evidence that pPGI is an important determinant of plastidic MEP pathway derived CKs (see below). Therefore, it is conceivable that the low net CO_2 assimilation rate, Jmax, TPU and ETR (**Figures 13, Figure 14**) (**Table 3**) as well as the reduced growth, energy and redox potential, and leaf starch content phenotypes of *pgi1* plants (**Figure 2, Figure 6, Figure 12, Figure 15**) can be ascribed, at least in part, to the low content of active forms of plastidic MEP pathway derived CKs. In this respect we must emphasize that, similar to *pgi1* plants, CKs-deficient plants have reduced size and accumulate low starch in their source leaves (Werner et al., 2001; Yang et al., 2003; Werner et al., 2008). That exogenous application of CKs partially reverted the reduced starch content phenotype of *pgi1* leaves (**Figure 17**) further supports the view that the low starch content phenotype of *pgi1* leaves can be partially the consequence of reduced pPGI-mediated production of CKs.

In Arabidopsis the prenyl group of tZ- and iP-type CKs is mainly produced through the plastidic MEP pathway, whereas a large fraction of the prenyl group of cZ derivatives is provided by the cytosolic MVA pathway (Kasahara et al., 2004). In *pgi1* leaves the most dramatic decrease in CKs content was found in the levels of CKs derived from the plastidic MEP pathway (iP- and tZ-type CKs), but not in the levels of cytosolic MVA-pathway dependent cZ-type CKs (**Table 4**). This would strongly indicate that pPGI is an important determinant of biosynthesis of CKs in plastids, but not in cytosol. Visible dif-

ferences between WT and *pgi1-2* leaves were found in the levels of iP- and tZ-type CKs (**Table 4**). While the level of iPRMP (the product of the first dimethylallyl diphosphate (DMAPP)-dependent step of CK biosynthesis) in *pgi1-2* leaves was 70% of that occurring in WT leaves, the level of tZRMP in *pgi1-2* leaves was only 32% of that occurring in WT leaves (**Table 4**). Noteworthy, while the lack of pPGI in *pgi1-2* leaves resulted in a 50% reduction of the level of tZ free base, the lack of pPGI did not affect the level of iP free base at all (**Table 4**). The relatively high level of iPRMP and iP in *pgi1-2* leaves could be explained by the transport of DMAPP from cytosolic MVA pathway into plastids (Kasahara et al., 2004) to increase the DMAPP pool accessible for plastid localized isopentenyltransferases (IPTs). Such mechanism of common isoprenoid precursors (e.g. isopentenyl diphosphate or DMAPP) exchange between the cytosol and plastids was proposed to explain MVA-derived contribution to plastidic biosynthesis of gibberellins (Helliwell et al., 2001). Another explanation for the relatively high level of iPRMP and iP in *pgi1-2* leaves can be that MVA-derived DMAPP serves as a substrate for AtIPT4, the cytosolic IPT isoform capable of *de novo* biosynthesis of iP, which can increase the overall iP cell pool (Kasahara et al., 2004). It is worth mentioning here that tZRMP can be formed by the iPRMP-dependent pathway through hydroxylation of its side chain by CYP735A (Takei et al., 2004), and also directly through the iPRMP-independent pathway, utilizing a yet unknown side-chain donor of terpenoid origin (Åstot et al., 2000) (**Figure 16**). In this last respect, it can be speculated that the side-chain precursor is 1-hydroxy-2-methyl-2-butenyl 4-diphosphate (HMBDP), which is downstream of GAP in the MAP pathway (**Figure 16**). The dramatic decrease of tZRMP and tZ levels can be thus explained as a combination of the lack of DMAPP needed to form iPRMP that is subsequently hydroxylated to tZRMP, together with the lack of a prenyl side chain precursor to form tZRMP.

Results presented in this work have provided evidence that pPGI is an important determinant of plastidic MEP pathway derived CKs likely as a consequence of its requirement in the production of GAP. GAP is the substrate for the initial reaction of the plastidic MEP pathway involved in the synthesis of isoprenoid derived molecules other than CKs such as gibberelins, abscisic acid, strigolactones, monoterpenes, carotenoids, tocopherols and prenylquinones (**Figure 16**), some of them mutually interacting and acting as important determinants of growth, photosynthetic capacity, starch content and energy status of the plant (Lichtenthaler et al., 1997; Schlüter et al., 2002; Phillips et

al., 2008; Oh et al., 2011; Pulido et al., 2012). It is thus likely that the reduced size and low starch content phenotypes of *pgi1* mutants are largely the consequence of changes in the overall plastidic MEP pathway-derived isoprenoid metabolism and its regulated processes. Needless to say, further research will be necessary to test this hypothesis.

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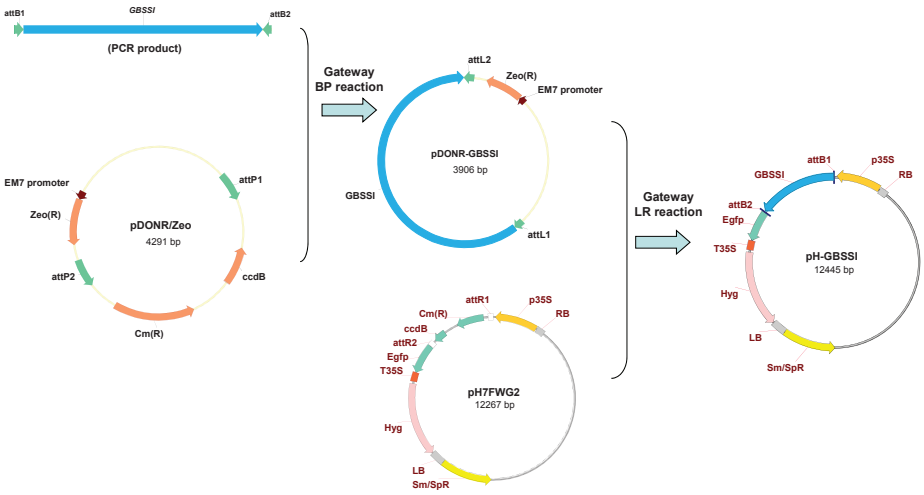
Supplemental Figure 1. Stages to construct the *35S-PGI1* and *35S-PGI1 plasmids necessary to produce *PGI1* and *PGI1** expressing plants.**

To obtain *PGI1* and *PGI1** cDNAs, 1.5 µg RNA extracted from WT and *pgi1-3* roots was reverse transcribed using polyT primers and the Expand Reverse Transcriptase kit (Roche) according to the manufacturer's instructions. PCR reactions were performed to generate *att*B-flanked PCR products using *PGI1* specific primers containing *att*B1 and *att*B2 recombinational cloning sites (*att*B1 primer: 5' GGGGACAAGTTTGTACAAAAAAGCAGGCTTAATGGCCTCTCTCTCAGGC-3'; *att*B2 primer: 5'-GGGGACCACTTTGTACAAGAAAGCTGGGTATTATGCGTACAGGTCATCCAC-3') to incorporate complete *att*B1 and *att*B2 sequences into the final PCR products.



Supplemental Figure 2. Stages to produce the *35S-GBSS-GFP construct**.

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1    A T G G C C T C T C T C T C A G G C C T A T A C T C T T C T T C T C C A T C T C T C A A A C C T G C  N92274

51   C A A A A A C C A T T C C T T T A A A G C A T T G C C G G C G C A A T C T C G A G A T T C C T T C T  er-105
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101  C T T T C C C A C A T A C C T C C A A A C C C A C C A A T C T A C C G T T G A C T C T C T C C T C G  er-105
101  C T T T C C C A C A T A C C T C C A A A C C C A C C A A T C T A C C G T T G A C T C T C T C C T C G  N92274

151  G C T C G T T C T G T G G C A C G C G A C A T T T C T C A T G C G G A T T C G A A G A A A G A G C T  er-105
151  G C T C G T T C T G T G G C A C G C G A C A T T T C T C A T G C G G A T T C G A A G A A A G A G C T  N92274

201  T C T T A A G G A T C C G G A T G C G T T G T G G A A G C G A T A C C T C G A T T G G T T T T A T C  er-105
201  T C T T A A G G A T C C G G A T G C G T T G T G G A A G C G A T A C C T C G A T T G G T T T T A T C  N92274

251  A G C A G A A A G A G C T T G G G T T G T A T C T G G A T A T T A G T C G G G T C G G A T T T A C C  er-105
251  A G C A G A A A G A G C T T G G G T T G T A T C T G G A T A T T A G T C G G G T C G G A T T T A C C  N92274

301  G A T G A G T T T G T G G C T G A A A T G G A G C C A A G G T T T C A A G C T G C G T T T A A G G C  er-105
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351  T A T G G A G G A T C T T G A A A A A G G G T C T A T A G C G A A T C C T G A T G A A G G A A G A A  er-105
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401  T G G T T G G A C A T T A C T G G C T T A G G A A C T C T A A G C T C G C A C C T A A G C C T A C T  er-105
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601  T T T G T C G C T G A G G C C T T G G C T C C T G A T A A T C C T C C A T T G A A G A T A A G A T T  er-105
601  T T T G T C G C T G A G G C C T T G G C T C C T G A T A A T C C T C C A T T G A A G A T A A G A T T  N92274

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701  G G C C A G A G C T G G C C T C G A C T T T A G T A G T T G T C A T C T C A A A G A G T G G A G G T  N92274

751  A C T C C T G A A A C T A G A A A T G G A C T A C T G G A A G T G C A G A A A G C A T T C C G T G A  er-105
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801  A G C T G G T C T G A A T T T C G C A A A A C A G G G T G T T G C A A T A A C C C A A G A G A A C T  er-105
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851  C A T T A C T G G A T A A T A C G G C A A G A A T T G A A G G T T G G C T A G C T A G A T T T C C T  er-105
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901  A T G T A C G A C T G G G T G G G T G G A A G A A C A T C A A T A A T G T C T G C A G T T G G T C T  er-105
901  A T G T A C G A C T G G G T G G G T G G A A G A A C A T C A A T A A T G T C T G C A G T T G G T C T  N92274

951  G C T T C C A G C A G C A C T A C A G G G G A T T A A T G T T A G G G A G A T G C T T A C T G G T G  er-105
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1001 C T G C T T T A A T G G A T G A G G C T A C T A G G A C A A C T T C G A T C A A G A A T A A C C C T  er-105
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1051 G C A G C G C T C T T A G C T A T G T G T T G G T A C T G G G C T T C C A A T G G C G T T G G T T C  er-105
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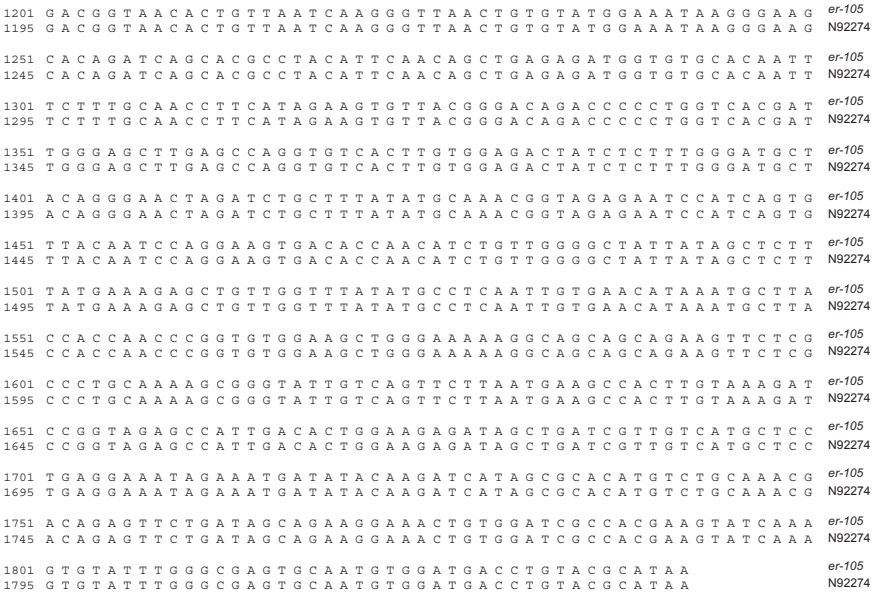
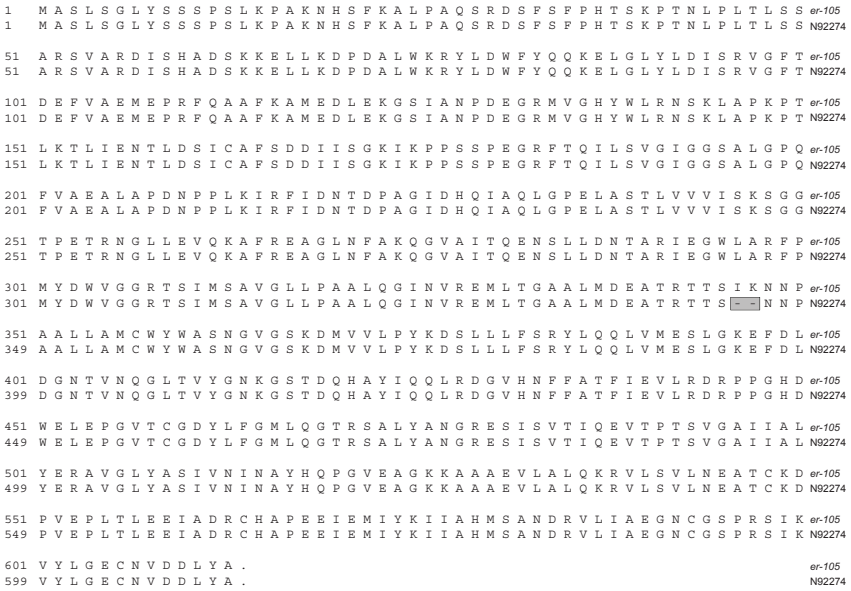


Supplemental Figure 3. Nucleotide sequences of complete pPGI encoding cDNAs obtained from *er-105* and N92274 (*pgi1-3*) plants.



Supplemental Figure 4. Amino acid sequences deduced from the nucleotide sequences shown in Supplemental Figure 3.

CHAPTER 2

Volatile compounds emitted by diverse phytopathogenic microorganisms promote plant growth and flowering through cytokinin action

ABSTRACT:

It is known that volatile emissions from some beneficial rhizosphere microorganisms promote plant growth. Here we show that volatile compounds (VCs) emitted by phylogenetically diverse rhizosphere and non-rhizhosphere bacteria and fungi (including plant pathogens and microbes that do not normally interact mutualistically with plants) promote growth and flowering of various plant species, including crops. In Arabidopsis plants exposed to VCs emitted by the phytopathogen Alternaria alternata, changes included enhancement of photosynthesis and accumulation of high levels of cytokinins (CKs) and sugars. Evidence obtained using transgenic Arabidopsis plants with altered CK status show that CKs play essential roles in this phenomenon, because growth and flowering responses to the VCs were reduced in mutants with CK-deficiency (35S:AtCKX1) or low receptor sensitivity (ahk2/3). Further, we demonstrate that the plant responses to fungal VCs are light-dependent. Transcriptomic analyses of Arabidopsis leaves exposed to A. alternata VCs revealed changes in the expression of light- and CK-responsive genes involved in photosynthesis, growth and flowering. Notably, many genes differentially expressed in plants treated with fungal VCs were also differentially expressed in plants exposed to VCs emitted by the plant growth promoting rhizobacterium Bacillus subtilis GB03, suggesting that plants react to microbial VCs through highly conserved regulatory mechanisms.

KEYWORDS: cytokinin; flowering; growth promotion; microbial volatile compounds; photoregulation; photosynthesis; plant-microbe interaction; starch

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GENERAL DISCUSSION

GENERAL DISCUSSION

Starch is the main storage carbohydrate in vascular plants. Synthesized in plastids, this reserve polysaccharide is an important determinant of plant growth and its relationship with the environment. It is widely assumed that the whole starch biosynthetic process resides exclusively in the chloroplast and segregated from the sucrose biosynthetic process that takes place in the cytosol (Neuhaus et al., 2005; Streb et al., 2009; Stitt and Zeeman, 2012). According to this classical view of starch biosynthesis starch is considered the end-product of a metabolic pathway that is linked to the Calvin-Benson cycle (CBC) by means of the plastidial phosphoglucose isomerase (pPGI). However, results presented in **Chapter 1** showing that mesophyll cells of mature leaves of the two *pgi1* null mutants impaired in pPGI accumulate ca. 10-15% of the WT starch content strongly indicate that the CBC is not directly connected to the starch biosynthetic pathway by means of pPGI. Kunz et al. (2010) reported that *pgi1-2* leaves exhibit substantial GPT activity as a consequence of the induction of *GPT2* and proposed that the occurrence of ca. 10% of the WT starch in *pgi1-2* leaves is ascribed to transport of G6P from the cytosol to the plastids of bundle sheath cells adjacent to the mesophyll and stomatal guard cells. Although this would indicate in principle that GPT2 could be involved in the incorporation of cytosolic G6P for its subsequent conversion into starch in *pgi1-2* mature leaves, results presented in **chapter 1** showing that the rate of starch accumulation in mature leaves of *pgi1* plants is comparable to that of leaves of *pgi1/gpt2* plants provide strong evidence that GPT2-mediated incorporation of cytosolic G6P into chloroplasts plays a minor role in the synthesis of starch in mesophyll cells of *pgi1* leaves. Our results showing that *pgi1* plants cultured under CL conditions are smaller than WT plants, whereas the near-starchless *aps1* and *pgm* plants display a WT growth phenotype in the same culture conditions, strongly indicate that (a) reduced starch turnover is not the reason of the slow growth phenotype of *pgi1* mutants, and (b) pPGI is an important determinant of plant growth.

pPGI is involved in the regeneration of G6P molecules in oxidative pentose phosphate pathway (OPPP) in heterotrophic organs and non-illuminated leaves. OPPP provides precursors for the synthesis of RNA, DNA and phenolic compounds such as aromatic amino acids, lignin, flavonoids and phytoalexins (Herrman, 1995). This metabolic pathway also provides NADPH necessary for biosynthetic redox reactions involved in

lipid biosynthesis and nitrogen assimilation (Kang and Rawsthorne, 1996; Bowsher et al., 2002; Bowsher et al., 2007; Jong et al., 2014), and for NADP-thioredoxin reductase (NTRC) dependent processes such as supply of reductant necessary for detoxifying hydrogen peroxide in the dark, and maintaining the redox homeostasis of plastids which in turn determines plant growth and development (Pérez-Ruiz et al., 2006; Lepistö et al., 2009; Kirchsteiger et al., 2012). Noteworthy, it has been reported that G6P metabolization within the OPPP is required for generating a signal that governs the regulation of root mediated acquisition of nitrogen and sulfur necessary for amino acid synthesis (Lejay et al., 2008). Also, previous reports have shown that, similar to plants impaired in pPGI, mutants impaired in other plastidic OPPP enzymes such as 6-phosphogluconolactonase display a reduced size phenotype (Xiong et al., 2009; Bussell et al., 2013). Therefore, it is conceivable that the reduced size of *pgi1* mutants is ascribed, at least in part, to impairments in some OPPP-dependent processes occurring in heterotrophic organs that are important for growth.

The low rates of growth and leaf starch accumulation of *pgi1* plants pointed to the possible occurrence of reduced photosynthetic capacity of *pgi1* leaves. In line with this presumption, results presented in **chapter 1** showed that the photosynthetic capacities of starch deficient *pgi1* plants are lower than that of WT plants. In addition, we observed that the adenylate energy charge and cellular redox potential of *pgi1* leaves are lower than those of WT leaves. This would indicate that starch deficiency of *pgi1* leaves is partially the consequence of either reduced CO_2 fixation capacity and/or low energy status and cellular redox potential, and not only the consequence of lack of pPGI-mediated flow between the CBC and the plastidic phosphoglucomutase (pPGM)- ADPG pyrophosphorylase (AGP)-starch synthases (SS) starch biosynthetic pathway.

Glyceraldehyde 3-phosphate (GAP) is a glycolytic and OPPP metabolic intermediate that acts as substrate for the initial reaction of the plastidic 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway involved in the synthesis of isoprenoids (Lichtenthaler et al., 1997; Phillips et al., 2008; Pulido et al., 2012). In non-illuminated leaves and heterotrophic organs GAP can be produced in the OPPP and glycolytic pathways involving pPGI. Therefore, pPGI could potentially act as a determinant for the synthesis of plastidic MEP-pathway derived isoprenoid compounds. Among different plastidic isoprenoid derived molecules, cytokinins (CKs) have been shown to act as major determinants of growth, energy status, starch content and photosynthesis in mature leaves (Synková et

al., 1999; Yang et al., 2003; Yaronskaya et al., 2006; Werner et al., 2008; Cortleven et al., 2011; Cortleven and Valcke, 2012; Erickson et al., 2014). Moreover, plastidic MEP pathway derived CKs are mainly synthesized in roots and transported to the aerial parts of the plant, where they regulate plant growth (Ko et al., 2014). The results presented in **chapter 1** showed that total levels of plastidic MEP pathway derived forms of CKs in *pgi1-2* leaves are low when compared with WT leaves, which provides evidence that pPGI is an important determinant of plastidic MEP pathway derived CKs. Moreover, exogenous application of CKs partially reverted the reduced starch content phenotype of *pgi1* leaves. Thus, the overall data show that pPGI is an important determinant of photosynthesis, energy status, growth and starch accumulation in mesophyll cells likely as a consequence of its involvement in the production of OPPP/glycolysis intermediates necessary for the synthesis of plastidic CKs in roots.

It is known that volatile emissions from some beneficial rhizosphere microorganisms promote plant growth. Furthermore, volatile compounds (VCs) from a number of microorganisms ranging from Gram-negative and Gram-positive bacteria to different fungi promote accumulation of exceptionally high levels of starch in leaves of mono- and di-cotyledonous plants (Ezquer et al., 2010; Li et al., 2011). To obtain insights into the mechanisms involved in the microbial VCs-mediated promotion of growth, starch accumulation and flowering, in **Chapter 2** we characterized Arabidopsis plants exposed to the VCs emitted by a number of beneficial and non-beneficial, phylogenetically diverse microorganisms (including plant pathogens). The results showed that blends of VCs emitted by all microorganisms tested promote starch accumulation, growth and flowering in various plant species, including crops of agronomic interest. In addition, we found that VCs emitted by the pathogen *Alternaria alternata* have positive effects on photosynthesis in Arabidopsis plants, which can be ascribed, at least partially, to very efficient use of light as a consequence of enhanced accumulation of photosynthetic pigments and improved ETR. As indicated above, it is known that CKs are major determinants of growth, energy status and photosynthesis in mature leaves (Cortleven and Valcke, 2012; Kieber and Schaller, 2013; Bahaji et al., 2015). Furthermore, these versatile hormones play important roles in flowering (Nishimura et al., 2004; Riefler et al., 2006; D´Aloia et al., 2011), modulation of sugar-induced anthocyanin accumulation (Guo et al., 2005; Das et al., 2012) and interaction of the plant with both biotic and abiotic factors (Argueso et al., 2012). Moreover, CKs promote starch accumulation in leaves

(Werner et al., 2008) most likely by regulating the expression of starch metabolism related genes (Miyazawa et al., 1999) and/or enhancing photosynthetic CO_2 fixation. Results presented in **chapter 2** showing that levels of plastidic MEP derived CKs in leaves of plants treated with *A. alternata* VCs are higher than in non-treated leaves would indicate that enhancement of CKs is involved in the VCs-promoted changes. This hypothesis is corroborated by the poor responses to VCs observed in 35S:CKX1 with CK-deficiency and the CKs signaling *ahk2/3* mutant. It should be noted that the first suggested level of diurnal MEP regulation is related to the CBC intermediate GAP (Pulido et al., 2012; Pokhilko et al., 2015). In VCs-treated leaves, GAP concentration is 2-fold higher than that of non-treated leaves, likely as a consequence of enhanced photosynthesis. Thus, accumulation of high levels of active MEP derived CKs in leaves of VCs-treated plants might be at least partly due to enhanced photosynthetic production of GAP and subsequent conversion into MEP-derived CKs.

Transcriptomic analyses of Arabidopsis leaves exposed to *A. alternata* VCs revealed changes in the expression of light- and CK-responsive genes involved in photosynthesis, growth and flowering. Taken together, these data strongly indicated that changes in VCs-exposed plants result from complex, transcriptionally regulated processes allowing the plant to acclimate to new environmental conditions in which light and CKs play important roles. Notably, many genes differentially expressed in plants treated with fungal VCs were also differentially expressed in plants exposed to VCs emitted by the plant growth promoting rhizobacterium *Bacillus subtilis* GB03, suggesting that plants react to microbial VCs through highly conserved regulatory mechanisms.

To obtain insights into the mechanisms involved in the *A. alternata* VCs promoted growth, photosynthesis and accumulation of CKs and starch, and to investigate the extent to which pPGI is involved in these responses, in **chapter 3** I explored the role of pPGI in the response of Arabidopsis to VCs emitted by *A. alternata*. The results obtained showed that *A. alternata* VCs promote growth and the accumulation of high levels of starch in mesophyll cells of *pgi1-2* plants as a consequence of the photosynthesis-driven enhancement of plastidic CK production in leaves, which, in turn, further promotes photosynthesis. This phenomenon is accompanied by the accumulation of stress-responsive amino acids such as alanine and GABA, which may represent a strategy for adaptation to the environmental conditions caused by exposure to VCs emitted by *A. alternata*. Moreover, *A. alternata* VCs promote changes in the expression of proteins

involved in photosynthesis, starch metabolism and growth that can account for the observed responses in *pgi1-2* plants. The overall data presented in **chapter 3** show that Arabidopsis plants can respond to VCs emitted by phytopathogenic microorganisms by triggering pPGI-independent mechanisms. The finding that leaves of *pgi1-2* plants exposed to fungal VCs accumulate exceptionally high levels of starch when the rate of photosynthesis increases conflicts with the widely accepted view that the whole photosynthesis-driven starch biosynthetic process occurs solely in the chloroplast by means of the CBC–pPGI-pPGM-AGP-SS pathway, and raises important questions regarding the basic mechanisms of starch biosynthesis in leaves exposed to VCs. On the other hand, this finding is consistent with the idea that the starch deficiency in *pgi1-2* leaves not exposed to VCs is partly due to the reduced CO_2 fixation capacity of this mutant, rather than only a consequence of the lack of pPGI-mediated flow between the CBC and the pPGM-AGP-SS starch biosynthetic pathways (Bahaji et al., 2015).

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CONCLUSIONES

CONCLUSIONES

1) pPGI es un determinante importante de la fotosíntesis, el crecimiento y la acumulación de almidón en células del mesófilo, probablemente debido a su participación en la generación de poder reductor necesario para la fijación de nitrógeno y/o precursores para la síntesis de aminoácidos aromáticos e isoprenoides plastidiales tales como las CKs.
2) El fenotipo de bajo almidón de los mutantes pPGI puede deberse no sólo a la falta de conexión entre el ciclo de Calvin-Benson y la ruta "PGM-AGP-SS" de síntesis de almidón sino también a una reducida capacidad fotosintética y una elevada actividad degradadora de almidón.
3) Existen importantes rutas de biosíntesis de almidón diferentes a la ruta pPGI-pPGM-AGP-SS que implican una conexión entre el cloroplasto y el citosol en células del mesófilo.
4) Mezclas de VCs emitidos por un amplio rango de bacterias y hongos filogenéticamente distantes (incluyendo patógenos de plantas) fomentan el crecimiento, floración y sobreacumulación de almidón.
5) El incremento de la fotosíntesis, crecimiento y floración debido a VCs emitidos por un hongo patógeno de plantas implica una compleja red de procesos regulados tanto a nivel transcripcional como post-transcripcional, en los que la luz y el incremento de la producción de CKs juegan un papel importante.
6) Las plantas han desarrollado la capacidad de reaccionar a VCs emitidos por diferentes microorganismos a través de mecanismos de regulación altamente conservados.
7) Los VCs emitidos por el fitopatógeno *A. alternata* activan o estimulan importantes rutas de biosíntesis de almidón independientes de la ruta CBC-pPGI-pPGM-AGP-SS, permitiendo que las células del mesófilo acumulen niveles de almidón excepcionalmente altos.
8) Los VCs emitidos por *A. alternata* activan/estimulan mecanismos independientes a pPGI que aumentan la fotosíntesis y crecimiento, probablemente debido a un incremento de la producción de CK plastidiales en hojas.

CONCLUSIONS

CONCLUSIONS

1) pPGI is an important determinant of photosynthetic CO_2 fixation, plant growth and starch accumulation in mesophyll cells likely as a consequence of its involvement in the generation of reducing power necesary for nitrogen fixation and/or precursors for the synthesis of aromatic amino acids and plastidic MEP pathway derived isoprenoid compounds such as CKs.
2) The low starch content phenotype of pPGI null mutants can be ascribed, at least in part, to reduced photosynthetic capacity and high starch breakdown activity, and not only to the lack of pPGI- mediated flow between the Calvin-Benson cycle and the pPGM-AGP-SS starch biosynthetic pathway.
3) There occur important starch biosynthetic pathway(s) different to the pPGI-pPGM-AGP-SS pathway involving a metabolic link between the cytosol and the chloroplast of mesophyll cells.
4) Blends of VCs emitted by a wide range of phylogenetically distant rhizosphere and non-rhizosphere bacteria and fungi (including plant pathogens) promote plant growth, flowering and accumulation of exceptionally high levels of starch.
5) Promotion of photosynthesis, growth and flowering by VCs emitted by a fungal plant pathogen involves a complex network of transcriptionally and post-transcriptionally regulated processes wherein enhancement of CKs production and light play important roles.
6) Plants have evolved the capacity to react to VCs emitted by different microorganism through highly conserved regulatory mechanism.
7) VCs emitted by the phytopathogen *A. alternata* activate/stimulate important CBC-pPGI-pPGM-AGP-SS independent starch biosynthetic pathways allowing the mesophyll cells of leaves to accumulate exceptionally high levels of starch.
8) *A. alternata* VCs activate/stimulate pPGI independent mechanisms that increase the photosynthesis and growth likely as a consequence of photosynthesis-driven enhancement of plastidic CK production in leaves.

APPENDIX

LIST OF ABBREVIATIONS

3PGA	3-phosphoglycerate
A/N-inv	Alkaline/neutral invertase
ABA	Abscisic acid
ADPG	ADPglucose
AGP	ADPG pyrophosphorylase
AGPP	ADPG pyrophosphatase
AMPK	AMP-activated protein kinase
An	Net photosynthetic CO2 fi xation rates
APS1	Small AGP subunit
BT1	Brittle1
CBC	Calvin-Benson cycle
CFM	Confocal fluorescence microscopy
Ci	Intercellular CO2 concentrations
CKs	Cytokinins
CL	Continuous light
Col-*0*	Columbia
cytPGI	Cytosolic phosphoglucose isomerase
cZ	cis-zeatins
DAS	Days after sowing
DMAPP	Dimethylallyl diphosphate
DW	Dry weight
DZ	Dihydroxy zeatin
er-105	Columbia with erecta-105 mutation
ETR	Photosynthetic electron transport rate
F6P	Fructose-6-phosphate
FW	Fresh weight
G1P	Glucose-1-phosphate
G6P	Glucose-6-phosphate
GAP	Glyceraldehyde 3-phosphate
GPT	G6P/Pi translocator
gs	Stomatal conductance
GWD	Water-dikinases

IPTs	Isopentenyltransferases
Jmax	Maximum rate of the electron transport
LD	Long day
MCF	Mitochondrial carrier family
MEP	2-C-methyl-D-erythritol 4-phosphate
MIVOISAP	MIcrobial VOlatiles Induced Starch Accumulation Process
MS	Murashige and Skoog
MVA	Mevalonate
NO	Nitric oxide
NPP	Nucleotide pyrophosphatase/phosphodiesterases
NTRC	NADP-thioredoxin reductase C
OPPP	Oxidative pentose-P pathway
pHK	Plastidial hexokinase
PPase	Alkaline pyrophosphatase
pPGI	Plastidial phosphoglucose isomerase
pPGM	Plastidial phosphoglucomutase
PPi	Inorganic pyrophosphate
PSII	Photosystem II
pSP	Plastidial starch phosphorylase
ROS	Reactive oxygen species
RSR1	Rice Starch Regulator1
RuBP	Ribulose-1,5-bisP
SD	Short day
SNF1	Sucrose non-fermenting
SnRK1	Sucrose non-fermenting-1-related kinase-1
SPS	Sucrose-phosphate synthase
SS	Starch synthase
SuSy	Sucrose Synthase
TEM	Transmission electron microscopy
TPU	Triose phosphate use
Trxs	Thioredoxins
tZ	trans-zeatin
UDPG	UDPglucose

UGP	UDPG pyrophosphorylase
Vcmax	Maximum carboxylation rate
VCs	Volatile compounds
Ws-2	WT Wasilewskija
WT	Wild-type
Φ_{NPQ}	Non-photochemical quenching
Φ_{Po}	Maximum quantum yields of PSII in the dark-adapted state
Φ_{PSII}	PSII operating efficiency

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1. Baslam, M., Baroja-Fernández, E., Ricarte-Bermejo, A., **Sánchez-López, Á.M.,** Aranjuelo, I., Bahaji, A., Muñoz, F.J., Almagro, G., Pujol, P., Galarza, R., Teixidor, P., Pozueta-Romero, J. (2016) Genetic and isotope ratio mass spectrometric evidence for the occurrence of starch degradation and cycling in illuminated Arabidopsis leaves. *Plant J.* (Submitted)

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