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Unravelling the mechanisms that improve photosynthetic performance of N_2-fixing pea plants exposed to elevated [CO_2]

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ABSTRACT

Although the predicted enhanced photosynthetic rates of plants exposed to elevated [CO_2] are expected to increase carbohydrate and plant growth, recent findings have shown a complex regulation of these processes. The aim of this study was to determine the effect of elevated [CO_2] on pathways leading to the main forms of leaf C storage (starch) and export (sucrose) and the implications of this increased [CO_2] on photosynthetic performance of exclusively N_2 fixing plants. For this purpose, exclusively N_2-fixing pea plants were exposed to elevated [CO_2] (1000 μmol mol^{-1} *versus* 360 μmol mol^{-1} CO_2). The data obtained highlighted that plants exposed to elevated [CO_2] were capable of maintaining hexose levels (involved in Rubisco down regulation) at control levels with the consequent avoidance of photosynthetic acclimation. More specifically, in plants exposed to elevated [CO_2] there was an increase in the activity of pathways involved in the main forms of leaf C storage (starch) and export (sucrose). Furthermore, the study highlighted that although starch content increased by up to 40% under elevated [CO_2], there was also an increase in the proteins and compounds involved in starch degradation. Such a finding, together with an increase in the activity of proteins involved in sucrose synthesis revealed that these plants up-regulated the sucrose synthesis pathway in order to meet the large nodule photoassimilate requirements. As a consequence, the study highlighted the relevance of controlling the activity of pathways that determine leaf cellular carbohydrate availability and how this is linked with C-demanding organs such as nodules.

1. Introduction

Plant photosynthetic performance is conditioned by changing environmental conditions. Most plants buffer these changes by adapting the activity of proteins involved in photosynthesis (Leakey et al., 2009). Another target point to balance leaf carbohydrate availability is the mobilization of photoassimilates (mainly in the form of sucrose) towards C-demanding organs and/or by retaining part of the photosynthate as starch (Gibon et al., 2009). The effect of atmospheric [CO_2] in photosynthetic performance has been studied extensively during recent decades (Leakey et al., 2009). Because the current [CO_2] in the atmosphere is generally limiting for C_3 photosynthesis, it has been suggested that the predicted [CO_2] increase will enhance the photosynthetic rate of plants (Farquhar et al., 1980). However, the initial stimulation has been described as being partially reversed in an acclimation process often referred to as "down-regulation" (Leakey et al., 2009). According to the model proposed by Krapp et al. (1993) and Moore et al. (1999), as a result of a larger atmospheric [CO_2] (C_a) there would be an increase in the photosynthetic rates of plants and consequently in the availability of a major leaf photoassimilate form such as sucrose. More specifically, the enhanced HK catalytic activity initiates the transduction response that induces the repression of the promoter activities of Rubisco and other photosynthetic genes (Krapp et al., 1993; Moore et al., 1999).

The leaf soluble sugar level is conditioned by the leaf's metabolic activity, storage processes, and the sink strength of other plant organs (Moore et al., 1999). Therefore, the leaf sucrose content may reflect the balance of C sink/source demand. In this sense, starch

Abbreviations: ADPG, ADP-glucose; A_n, net photosynthetic rate; A_{360}, A_n determined at 360 μmol mol^{-1} CO_2; A_{1000}, A_n determined at 1000 μmol mol^{-1} CO_2; C_a, atmospheric [CO_2]; Gluc, glucose; Gluc1P, glucose-1-phosphate; Gluc6P, glucose-6-phosphate; Fruc, fructose; Fruc6P, fructose-6-phosphate; Fruc-2,6bP, fructose-2,6-bisphosphate; HK, hexokinase; PGM, phosphoglucomutase; PHI, phosphohexose isomerase; Pi_{chl}, chloroplastic phosphate; Pi_{cyt}, cytosolic phosphate; N_{red}, reduced N content; SP, starch phosphorylase; Suc, Sucrose; TOA, total organic acid; TP, triose phosphate; TSP, total soluble protein; TSS, total soluble sugar; UDPG, UDP-glucose; UDPG-PPi_{ase}, uridine diphosphoglucose pyrophosphorylase; α-Amy, α-amylase; β-Amy, β-amylase; 3PGA, 3 phosphoglyceric acid.

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has also been proposed as a buffer for sucrose metabolism that might minimize leaf sucrose cycling (Moore et al., 1999). Although the synthesis of sucrose and starch takes place in the cytosol and chloroplast, the C fluxes through these pathways are highly regulated in order to avoid the inhibition of photosynthetic activity (Stitt et al., 2010). Starch synthesis in leaves has been traditionally considered to be exclusive to chloroplasts and the synthesis of sucrose exclusive to the cytosol (Neuhaus et al., 2005). According to these studies, starch is considered as the end product of a unidirectional pathway where ADP-glucose pyrophosphorylase (APG) exclusively catalyses the synthesis of ADP-glucose (ADPG). However, the pathway leading to starch synthesis has been recently questioned (Stitt et al., 2010). Studies conducted by Baroja-Fernández et al. (2004) observed that ADPG can also be synthesized in the cytosol by sucrose synthase and afterwards imported into the chloroplast for starch synthesis.

Previous studies have highlighted that responsiveness of plants to elevated [CO_2] is restricted under N limiting conditions. The negative acclimation of photosynthesis may be the result of lowered leaf N content due to limitations in soil N availability conditions (Theobald et al., 1998). Indeed, it has been suggested that a persistent increase in plant biomass production under elevated [CO_2] can only be maintained by an increase in N uptake (Soussana and Hartwig, 1996). Furthermore, there is evidence that the carbohydrate-mediated repression of photosynthetic genes is more severe in nitrogen deficient plants (Aranjuelo et al., 2013). According to some authors (Serraj et al., 1998; Rogers et al., 2006), because legumes are capable of fixing atmospheric N_2 they will have access to atmospheric N_2 and they will have an advantage in plant growth over non-N_2-fixing plants, especially in N limited soils. Moreover, legumes have an extra sink for additional C to be transferred to nodules, thus enhancing N_2 fixation (Udvardi and Day, 1997), which avoids leaf carbohydrate accumulation and therefore acclimation of photosynthesis (Erice et al., 2011).

Although Aranjuelo et al. (2013) showed that exclusively N_2 fixing pea plants were capable of maintaining improved photosynthetic rates, in contrast to the photosynthetic acclimation observed in NO_3^--fed plants under high [CO_2], the role played by nodules in this remains unclear. The aim of this study is to characterize nodule C sink demand and its implications in regulation of the synthesis of two major leaf carbohydrates (sucrose and starch) and to ascertain the mechanisms involved in this enhanced performance under elevated [CO_2]. For this purpose, nodule activity together with enzymes and intermediate compounds involved in the synthesis of leaf sucrose and starch were monitored in exclusively N_2 fixing pea plants exposed to elevated (1000 μmol mol^{-1}) and ambient (360 μmol mol^{-1}) [CO_2].

2. Materials and methods

2.1. Plant material and experimental design

The experiment was conducted with exclusively N_2-fixing pea (*Pisum sativum* L. cv Frilene) plants, inoculated with *Rhizobium leguminosarum* biovar viciae strain NLV8, which is hup-, grown in 2.5 L plastic pots (one plant per pot) filled with 3:2 (v/v) vermiculite-perlite. Sixteen plants per treatment were grown in controlled-environment chambers (Heraeus-Votsch HPS-500, Norrkoping, Sweden) at 25/18 °C (day/night) with a photoperiod of 16 h of 480 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) and a relative humidity of 70/80% (day/night). The cabinets provided two independent T-RH combined probes (41372VC/VF RM Young Co., Traverse City, MI, USA) connected to an external microprocessor (LI-1000, LiCor, Lincoln, Nebraska, USA). Plants were irrigated with a drip system, with N-free nutrient solution (Rigaud and Puppo, 1975). Half of the randomly selected plants were placed into an elevated [CO_2] cabinet where they were exposed to 1000 μmol mol^{-1}, whereas the other half were placed in a cabinet with ambient [CO_2] (≈360 μmol mol^{-1}). CO_2 bottles were provided by Praxair (Pamplona, Spain). Air entered the cabinets from a compressor installed at the top of the building, and was filtered by four air filters (coarse-5 μm and 1 μm Ø particle and 0.01 μm Ø particle physical filters and a charcoal chemical filter) to prevent anomalous components. Cabinets were equipped with an infrared CO_2 analyser (polytron-IRGA, Dragäer, Lübeck, Germany) connected to a microprocessor located inside the cabinet. [CO_2] was analyzed and controlled every second. All the determinations were conducted after 4 weeks of exposure to elevated [CO_2] conditions.

2.2. Sampling and plant growth determinations

Plant sampling was always carried out 5 h after the onset of the photoperiod. For plant growth determinations, samples were dried at 70 °C for 48 h in order to obtain the dry biomass (DM).

2.3. Gas exchange determinations

Fully expanded apical leaves were enclosed in a Li-Cor 6200 gas exchange portable photosynthesis system (Li-Cor, Lincoln, Nebraska, USA). Net photosynthesis (A_n) was determined according to Long and Hallgreen (1985) in all the plants at 360 (A_{360}) and 1000 (A_{1000}) μmol m^{-2} s^{-1} CO_2. Stomatal conductance (g_s) was determined as described by Harley et al. (1992).

2.4. Metabolic compound determinations

2.4.1. Leaf determinations

Rubisco activity and content was determined in leaves that were harvested at mid-morning and immediately plunged into liquid nitrogen. The samples were stored at −80 °C, awaiting analysis. The leaf tissue was powdered in liquid nitrogen and homogenized in a cold mortar with an extraction buffer containing 100 mM Bicine-NaOH (pH 7.8), 10 mM $MgCl_2$, 1 mM Na_2-EDTA, 5 mM DTT and 2% PVPP (Aranjuelo et al., 2005). An aliquot of the extract was used to determine the chlorophyll content (Arnon, 1949). Another aliquot was clarified by centrifugation at 20,000 × *g*, and used to determine enzyme activity by measuring the oxidation of NADH at 340 nm (Sharkey et al., 1991). For Rubisco initial and total activity assays, an NADH-coupled spectrophotometric procedure was followed (Pérez et al., 2005). The activation state was calculated by considering the initial activity as a percentage of the total activity.

Rubisco protein content was determined according to Aranjuelo et al. (2005). Aliquots of the dissociated extracts, containing 9 μg of protein, were applied to each well. The gels were stained in 0.1% (w/v) Coomassie blue dissolved in 5:5:2 (v/v/v) water–methanol–acetic acid overnight, and subsequently destained in 12.5% (v/v) isopropanol and 10% (v/v) acetic acid. Finally, the gels were scanned with a BioRad GS-700 densitometer. The protein content was determined with Molecular Analyst software (BioRad). Rubisco activity determinations (with the exception of Rubisco and glycolate oxidase) were conducted in a buffer containing 50 mM MPOS (pH 7.2), 20 mM KCl, 5 mM $MgCl_2$, 1 m MK_2-EDTA and 5 mM DTT according to Scott and Kruger (1995). After centrifugation at 25,000 × *g* for 20 min the supernatant was filtered through a 5 mL Bio GEL P6DG (BioRad) column and centrifuged at 180 × *g* for one minute. The supernatant was equilibrated with a solution containing 50 mM MOPS (pH 7.2), 20 mM KCl and 5 mM $MgCl_2$. The enzyme determinations were carried out