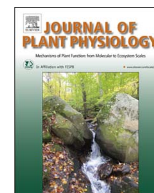
Journal of Plant Physiology 218 (2017) 144–154

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph



Research Paper

Leaf carbohydrates influence transcriptional and post-transcriptional regulation of nocturnal carboxylation and starch degradation in the facultative CAM plant, *Mesembryanthemum crystallinum*☆

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ARTICLE INFO

Keywords:
Crassulacean acid metabolism
Starch degradation
Metabolic regulation

ABSTRACT

Nocturnal degradation of transitory starch is a limiting factor for the optimal function of crassulacean acid metabolism and must be coordinated with phospho*enol*pyruvate carboxylase (PEPC)-mediated CO_2 uptake to optimise carbon gain over the diel cycle. The aim of this study was to test the hypothesis that nocturnal carboxylation is coordinated with starch degradation in CAM via a mechanism whereby the products of these pathways regulate diel transcript abundance and enzyme activities for both processes. To test this hypothesis, a starch and CAM-deficient mutant of *Mesembryanthemum crystallinum* was compared with wild type plants under well-watered and saline (CAM-inducing) conditions. Exposure to salinity increased the transcript abundance of genes required for nocturnal carboxylation, starch and sucrose degradation in both wild type and mutant, but the transcript abundance of several of these genes was not sustained over the dark period in the low-carbohydrate, CAM-deficient mutant. The diel pattern of transcript abundance for PEPC mirrored that of PEPC protein, as did the transcripts, protein, and activity of chloroplastic starch phosphorylase in both wild type and mutant, suggesting robust diel coordination of these metabolic processes. Activities of several amylase isoforms were low or lacking in the mutant, whilst the activity of a cytosolic isoform of starch phosphorylase was significantly elevated, indicating contrasting modes of metabolic regulation for the hydrolytic and phosphorylytic routes of starch degradation. Externally supplied sucrose resulted in an increase in nocturnal transcript abundance of genes required for nocturnal carboxylation and starch degradation. These results demonstrate that carbohydrates impact on transcriptional and post-transcriptional regulation of nocturnal carboxylation and starch degradation in CAM.

1. Introduction

In plants with crassulacean acid metabolism, a diel separation of carboxylation processes mediated by phospho*enol*pyruvate carboxylase (PEPC) and ribulose-1, 5-bisphosphate carboxylase/oxygenase (RUBISCO) optimizes photosynthetic performance and carbon gain in water-limited environments. During CAM, malic acid is accumulated overnight via PEPC-mediated carboxylation and is subsequently broken down to release CO_2 that is fixed by RUBISCO during the following day behind closed stomata, thereby conserving water and resulting in water-use efficiencies that exceed those of C_4 and C_3 plants by at least 3- and 6-fold respectively (Borland et al., 2009). The temporal separation of C_3 and C_4 carboxylation processes that defines CAM provides plasticity for optimizing carbon gain and water use in response to changing environmental conditions by extending or curtailing the period of net CO_2 uptake over a 24 h period (Dodd et al., 2003). Thus, efficient operation of the pathway and the ecological success of CAM are based on mechanisms that synchronize the supply and demand for carbon whilst maintaining photosynthetic plasticity over the 24 h CAM cycle (Borland and Taybi, 2004).

Circadian control of carbon flux through PEPC is generally regarded as a key component underpinning the diel separation of carboxylation processes that define CAM (Nimmo, 2000). PEPC is activated at night via phosphorylation of a serine residue near the N-terminus of the protein that renders the enzyme more sensitive to PEP and the positive effectors, glucose-6-P and triose-P, and less sensitive to the allosteric

☆ This research was supported by grants from the Natural Environment Research Council, UK (to AMB, TT), and the National Science Foundation (IBN-0196070 and DBI-9813360) and the Nevada Agricultural Experiment Station (to JCC).
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http://dx.doi.org/10.1016/j.jplph.2017.07.021
Received 10 May 2017; Received in revised form 19 July 2017; Accepted 28 July 2017
Available online 05 August 2017
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inhibitor, malate (Nimmo et al., 1986; Chollet, 1996). Reversible phosphorylation of PEPC is catalysed by a dedicated Ser/Thr protein kinase (PEPc-kinase, PPCK), which in turn is regulated at the transcriptional level by the circadian clock (Hartwell et al., 1999; Taybi et al., 2000). Circadian control of the degradation of PPCK towards the end of the night might enable plants to anticipate dawn and ensure a rapid inactivation of PEPC at the start of the day, thereby avoiding futile cycling of malate synthesis and decarboxylation. However, PEPC activation status can be modified by leaf metabolic status, so that *Ppck* transcripts can persist beyond the start of the photoperiod if cytosolic malate is maintained below a threshold concentration (Borland et al., 1999). Because diel changes in malate content are a distinguishing feature of CAM, malate-elicited feedback inhibition of *Ppck* expression is thought to provide an effective means of fine-tuning CO_2 uptake over the day/night cycle in response to changes in environmental conditions (Borland and Taybi 2004). Thus, integration of circadian and environmental signals appears necessary for achieving the synchronization and plasticity of metabolism that is inherent to CAM.

PEPC-mediated carboxylation is intimately linked to the diel turnover of starch as the amount of CO_2 taken up during the night depends on the availability of C_3-carbon substrate (PEP) produced from the nocturnal degradation of starch accumulated during the day (Cushman et al., 2008a; Ceusters et al., 2014). The mechanisms and molecular targets for coordinating the reciprocal diel cycling of malate and carbohydrates that define CAM remain to be elucidated but, with analogy to the regulation of PEPC described above, starch degradation can be hypothesized to be subject to both circadian and metabolite control at transcriptional and posttranscriptional levels. In the C_3 model plant *Arabidopsis thaliana*, day/night and circadian oscillations of transcripts encoding enzymes of starch degradation have been reported (Harmer et al., 2000; Schaffer et al., 2001; Smith et al., 2004) and it seems that the circadian clock plays a central role in coordinating day/night starch turnover with growth and export (Weise et al., 2006; Graf et al., 2010; Graf and Smith, 2011). Posttranscriptional regulation has also been proposed to be important for timing starch breakdown in *A. thaliana* (Stitt and Zeeman, 2012), and the products of starch degradation have been implicated in sugar-mediated gene regulation of carbohydrate turnover, photosynthesis, and growth (Caspar et al., 1985; Corbesier et al., 1998; Stitt and Zeeman, 2012). For CAM, previous work has shown that the induction of CAM in *Mesembryanthemum crystallinum* by exposure to salinity is accompanied by increased activities of a range of starch-degrading enzymes implicated in both the hydrolytic and phosphorylytic routes of starch degradation (Paul et al., 1993). Moreover, robust circadian control of the transcript abundance of several genes implicated in carbohydrate turnover and chloroplast transport of C-metabolites has been reported to accompany CAM induction in *M. crystallinum* (Häusler et al., 2000; Dodd et al., 2003; Kore-eda et al., 2005; Cushman et al., 2008b). However, the influence of metabolite control on transcriptional and posttranscriptional regulation of starch degradation and its coordination with PEPC activation remains to be established. A further question relates to the levels of control exerted over the different genes and enzymes implicated in nocturnal starch degradation, given that C_3 and CAM plants appear to use different enzymatic routes to break starch down (Borland et al., 2009; Weise et al., 2011). Starch degradation is believed to be initiated by the concerted action of phosphoglucan water dikinase (PWD) and glucan water dikinase (GWD) disrupting the starch granules (Ritte et al., 2002; Kötting et al., 2005; Kötting et al., 2010). In *A. thaliana*, the disrupted starch granules are then broken down *via* a hydrolytic route which is catalysed by α and/or *β* amylases (αAMY, *β*AMY) with maltose as the major product that is exported from the chloroplast (Smith et al., 2005; Weise et al., 2005), although αAMY was shown to not be necessary for starch degradation in *A. thaliana* (Yu et al., 2005). In the cytosol, maltose is converted to sucrose *via* the concerted action of disproportionating enzyme (DPE2) and a cytosolic α-glucan phosphorylase (STP2) (Chia et al., 2004; Lu et al., 2006). In contrast, chloroplasts of CAM-performing *M. crystallinum* have been shown to predominantly export glucose-6-P (Neuhaus and Schulte, 1996; Kore-eda and Kanai, 1997), indicating that starch is degraded via the phosphorylytic route using chloroplastic α-glucan phosphorylase (STP1). Thus, in the CAM system, metabolite control of transcriptional and posttranscriptional regulation of the phosphorylytic route of starch degradation and its coordination with PEPC activation might be predicted to differ from that of the hydrolytic route.

The aim of the work presented here was to test the hypothesis that the products of starch degradation coordinate and regulate nocturnal carboxylation and starch degradation at both transcriptional and post-transcriptional levels in the CAM system. To test this hypothesis, a starch and CAM-deficient mutant of *M. crystallinum* (Cushman et al., 2008a) was compared with wild-type plants under well-watered and saline (CAM-inducing) conditions. Diel transcript abundances of genes implicated in PEPC activity and activation (*Ppc1, Ppck1*), the initiation of starch degradation (*Pwd1, Gwd1*), the hydrolytic route of starch degradation (*αAmy1*, *βAmy1, Dpe2, Stp2*), the phosphorylytic route of starch degradation (*Stp1*), and sucrose degradation (vacuolar invertase *V-Inv1*; sucrose synthase *Ssy1*) were compared in wild type and mutant under control and saline conditions. Diel patterns of protein abundance and activity were monitored in parallel for selected enzymes. Lastly, the transcript abundances of *Ppc1* and *Ppck1*, together with those of selected genes implicated in starch degradation were monitored over the day/night cycle in detached leaves fed with sucrose in order to directly assess the impact of sugars on the coordinated expression of these genes.

2. Material and methods

2.1. Plant material

Seeds of wild type and a starch-deficient *pgm* mutant (351) of the common ice plant (*Mesembryanthemum crystallinum*) (Cushman et al., 2008a) were germinated in soil in a growth chamber under a 12-h light (26 °C)/12-h dark (18 °C) cycle. Ten-day-old seedlings were transplanted individually into 0.5 l plastic pots containing soil (John Innes 2) and irrigated once daily with water. Plants were grown under fluorescent lighting of 400 μmol m^{-2} s^{-1} at plant height for the first 4 wk, then under 200 μmol m^{-2} s^{-1} to limit CAM expression, particularly in the mutant compared to wild type. Once per week, plants were watered with nutrient supplement (Phostrogen, UK). Four-week-old plants were subjected to salt treatment by watering daily for 10 d with about 50 ml of 400 mM NaCl solution per plant. The fourth pair of leaves was harvested from three plants of the wild type and the mutant every 4 h for 24 h, frozen in liquid-N_2, and stored at −80 °C. Sucrose feeding experiments were conducted on leaves detached from plants that had been treated with salt as above for 10 d. Fourth leaf pairs were excised, weighed, and placed in either water for the controls or in 0.25 M sucrose solution (Chiou and Bush, 1998). Sugar treatments began after 3 h in the photoperiod and leaves were maintained in sugar solutions during the full duration of the experiment. Leaves were sampled every 4 h over 24 h beginning at 8 h of incubation in sucrose solution. Leaves were then frozen in liquid N_2 and stored at −80 °C.

2.2. Extraction and measurement of carbohydrates and organic acids

Carbohydrates and organic acids were extracted by adding 1 g of frozen, powdered plant material to 5 ml of 80% methanol and incubating at 80 °C for 1 h. Titratable acidity was determined by titrating 1 ml of the methanol-soluble leaf extract with 1 mM NaOH to the pink phenolphthalein end point. Total soluble sugars were measured using the colorimetric phenol-sulphuric acid test of Dubois et al. (1956). For determination of starch content, the pellet remaining after methanol extraction was washed three times with 1 ml of 80% methanol and three times with 1 ml of distilled water, and then resuspended by

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