Carbon-use efficiency in green sinks is increased when a blend of apoplastic fructose and glucose is available for uptake.

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Abstract

Understanding how green sink strength is regulated in planta poses a difficult problem because non-structural carbohydrate (NSC) levels can have integrated, simultaneous feedback effects on photosynthesis, sugar uptake, and respiration that depend on specific NSC moieties. Photosynthetic gametophytes of the fern Ceratopteris richardii provide a simple land plant model to assess how different NSCs imported from the apoplast of intact plants affect green sink strength. Sink strength was quantified as the amount of exogenous sugar that plants grown in low light depleted from their liquid media, and the relative contributions of carbon assimilation by photosynthesis and sugar uptake was estimated from stable isotope analysis of plant dry mass. Gametophytes absorbed fructose and glucose with equal affinity when cultured on either hexose alone, or in the presence of an equimolar blend of both sugars. Plants also depleted sucrose from the surrounding media, although a portion of this disaccharide that was hydrolysed into fructose and glucose by putative cell wall invertase activity remained in the media. The δ^{13}C in plant dry masses harvested from sugar treatments were all close to −18‰, indicating that 25–39% of total plant carbon was from C3 photosynthesis (δ^{13}C = −29‰) and 61–75% was from uptake of exogenous sugars (δ^{13}C = −11‰). Carbon-use efficiency (i.e. carbon accumulated/carbon depleted) was significantly improved when plants had a blend of exogenous sugars available compared with plants grown in a single hexose alone. Plants avoided complete down-regulation of photosynthesis even though a large excess of exogenous carbon fluxed through their cells.

Key words: disaccharide, fern gametophyte, hexose, mixotrophy, monosaccharide transporter.

Introduction

The movement of sugar from source to sink is a basic paradigm in plant physiology. However, limitations to non-structural carbohydrate (NSC) transport are increasingly appreciated for their role in regulating NSC balance, from cells to tissues to whole plants (Wardlaw, 1990; Roitsch et al., 2000; Bledsoe and Orians, 2006; Bansal and Germino, 2008; McCormick et al., 2009; Sala et al., 2010). Additionally, sugar transport is commonly from one green tissue to another, and sugar sensing of different NSC moieties in green tissues can result in dynamic feedback effects on photosynthesis (Furbank et al., 1997; Paul and Foyer, 2001; Rolland et al., 2006) and respiration (Amthor, 1991; Lewitus and Kana, 1994; Gandin et al., 2009). However, a clear understanding of how carbohydrate transport, photosynthesis, respiration, and NSC balance in green sinks respond to sugar delivery is hindered by the difficulty of experimentally manipulating sugars immediately around sink cells in planta.

Many plant sinks lack symplastic connection to adjacent source tissues and must import extracellular sugars from their cell wall space using membrane transporters (Renault et al., 1992; Patrick and Offler, 2001). Sucrose is generally...
delivered to the apoplast, but this disaccharide is frequently hydrolysed into fructose and glucose by cell wall invertases (cwINVs) prior to hexose uptake into sink cells (LaLonde et al., 2003; Sherson et al., 2003; Roitsch and González, 2004). The relative efficiency of these steps can provide an important regulatory control point to modulate sink strength (Renault et al., 1992; Ruan et al., 1997; Roitsch et al., 2000; Fridman et al., 2004; Carrari and Fernie, 2006). The hydrolysis of sucrose within the apoplast, accompanied by the continued delivery of new sucrose into the apoplast, means that sucrose, fructose, and glucose will all be present in the apoplast at the same time. This makes it difficult to know how each sugar affects the balance between sugar uptake from the apoplast, and photosynthesis and respiration within sink cells.

Fern gametophytes allow the effects of different extracellular sugar types on green sinks to be determined in a land plant. Although these primitive plants are non-vascular, many regulatory aspects of NSC transport and metabolism have a deep evolutionary history that predates the evolution of phloem (Graham and Wilcox, 2000; Johnson et al., 2006; Johnson and Thomas, 2007; Vargas and Salerno, 2009). Bathing whole gametophytes in exogenous sugars induces mixotrophy, a combined strategy of auto- and heterotrophy by CO₂ photoassimilation and sugar uptake, respectively (Miller and Miller, 1961; Cordle et al., 2007; Alongi et al., 2009). Consequently, gametophytes growing in media containing exogenous sugar can effectively act simultaneously as a carbon source and carbon sink. Compared with transport processes in vascular plants, gametophyte mixotrophy is most similar to post-sieve element transport into symplastically isolated green sinks (Patrick, 1997) because carbohydrates in the bathing media must cross the apoplast of non-vascular cells. An advantage to using whole gametophytes to study these transport processes is that the apoplastic carbohydrate content around the cells of intact plants can be controlled non-invasively. This approach also eliminates source limitation processes as a potential rate-limiting influence on transport while allowing the interaction between concurrent source–sink behaviour in green sink cells to be studied more directly.

This investigation used fern gametophytes to address two main questions. First, does the type of extracellular sugar(s) made available in the cell apoplast determine green sink strength? Previous work with gametophytes of Ceratopteris richardii tentatively supported the hypothesis that apoplastic sucrose might be hydrolysed by putative cwINVs into fructose and glucose, but then mainly glucose alone was imported (Alongi et al., 2009). Functional characterization of sugar uptake in other plants suggests that fructose is underutilized, especially when glucose is available instead (Krook et al., 2000; Sherson et al., 2003; Kretzschmar et al., 2007), so green sink strength might depend mainly on the availability of exogenous glucose alone. However, a high-affinity fructose transporter has now been identified in Arabidopsis thaliana (Klepek et al., 2010). Consequently, the possibility that gametophyte sink strength can also depend on the presence of fructose, with or without glucose present, was explicitly examined in the sugar uptake experiments reported here.

Second, this study asked whether the balance between simultaneous carbon source and carbon sink behaviour in a green sink depended on the type of extracellular sugar(s) made available in the apoplast. The answer to this question depends on potential links between carbon assimilation by sugar uptake and photosynthesis, and on carbon loss by respiration. For example, mixotrophic tissues might be expected to have a reduced need for sugar uptake (i.e. a lower sink strength) when conditions are favourable for photosynthesis. Alongi et al. (2009) previously showed that incremental increases in light flux decreased uptake of extracellular glucose in C. richardii, suggesting that an inverse relationship exists between photosynthesis and glucose sink strength. However, sugar sensing can potentially alter rates of photosynthesis at the level of photochemistry (e.g. by enhancing chlorophyll synthesis) independent of changes in light availability (Lewitus and Kana, 1994; Furbank et al., 1997). Green sink strength could consequently be altered indirectly, through changes in photosynthesis, even when plants are grown at a single light level. Moreover, the response should depend on the degree to which specific carbohydrates elicit feedback effects on photosynthesis and respiration.

This study is based on gametophytes of C. richardii, an annual fern with a fast life cycle (Hickok et al., 1995). Total carbon and NSC concentrations were determined for plants grown under low light with several different exogenous sugars available. Stable isotopes were used to determine the proportion of carbon in gametophytes derived from their own C3 photosynthesis or uptake of exogenous C4 sugars derived from Saccharum. The amount of exogenous sugar depleted from the bathing media was also determined. This allowed us to determine the sink strength and carbon-use efficiency of mixotrophic plants with different availabilities of apoplastic sugars.

Materials and methods

Plant growth conditions and harvest

Twenty-five sterile 125 ml Erlenmeyer flasks were each prepared with 50 ml of liquid basal salts media (pH 6.0) made from stock solutions, based on standard culture conditions recommended for C. richardii gametophytes (www.c-fern.org). Twenty of these were augmented with one of four sugar treatments: 60 mM sucrose, 60 mM fructose, 60 mM glucose, or 30 mM fructose plus 30 mM glucose (hereafter referred to as ‘FG’). These sugar concentrations cause significant effects on C. richardii gametophyte development without causing unwanted osmotic effects (Cordle et al., 2007; Alongi et al., 2009). All media were autoclaved since sucrose does not hydrolyse at pH 6.0 (George et al., 2007). The non-sugar control and each of the four sugar treatments were thus replicated five times, with the flask as the unit of replication.

Two batches of RNW1 spores (Carolina Biological Supply, Burlington, North Carolina, USA) were weighed (70 mg and 75 mg, respectively), surface sterilized with 0.88% sodium hypochlorite (commercial bleach) solution and washed twice in sterile distilled water. Each batch of spores was sown into media flasks (∼70 mg divided evenly into 12 flasks and ∼75 mg divided equally
into 13 flasks). Sowing was accomplished by suspending spores in water with a sterile transfer pipette and adding an equal number of drops to each flask. Spores were frequently resuspended during this process by re-evacuating the pipette to ensure approximately even spore densities in each drop. Flasks were placed under continuous fluorescent lighting (~125 \( \mu \text{mol} \text{ M}^{-2} \text{ S}^{-1} \)) for 48 h to stimulate spore germination and then moved to continuous low-intensity fluorescent lighting (~10 \( \mu \text{mol} \text{ M}^{-2} \text{ S}^{-1} \)) at 28–29°C in an environmental chamber for an additional 10 d. Light levels outside flasks at plant culture height were quantified with a LiCor LI 250A quantum sensor (Li-Cor Biosciences, Lincoln, NE, USA).

Twelve days after sowing spores, the contents of each flask were poured through a 70-\( \mu \text{m} \) cell strainer (Beckton-Dickinson Co., Franklin, NJ, USA) to separate the plants from the media. A small number of gametophytes (<0.5 mg fresh weight) were removed from each sample and stored as 50% ethanol for subsequent photography and image analysis. Plants remaining in each cell strainer were then immediately microwaved for 30–45 s to stop carbohydrate metabolism (Hoch et al., 2002) and placed in a drying oven at 70°C for 24 h to determine the dry mass. Dried plants were then ground into a fine powder using a glass mortar and pestle. The medium from each flask was also weighed (±1 mg) to account for any evaporative loss plus uptake by growing gametophytes; an aliquot of each sample was subsequently processed for soluble sugar content.

Image analyses

Plant area was estimated by photographing three gametophytes selected at random from each of three different treatment flasks (i.e. \( n = 3 \), each with three sub-replicates). Digital images were made on a Leica ATC 2000 microscope (Leica Microsystems, Wetzlar, Germany) equipped with a Canon PowerShot (Canon, Inc., Tokyo, Japan), and plant area was determined with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

During the experiment, plants growing in sugar appeared darker green than plants growing in control media without sugar. Prior to committing samples to NSC and carbon content analyses (see below), dried tissues were photographed to quantify these apparent differences. Digital images of each ground tissue sample were taken under uniform epi-illumination and at the same angle and distance using a Canon EOS Rebel (Canon, Inc., Tokyo, Japan) mounted on a tripod. The camera was operated in manual mode and all exposures were made at f 32 at 1/5 s. In ImageJ, colour was converted to greyscale, and the mean grey value for a ~1 cm\(^2\) square at the centre of each image was analysed to determine average grey value (as a proxy for chlorophyll content). This assumption was corroborated by chlorophyll extraction from gametophytes in a different study that showed that plants grown with NSC analyses

Total NSC is defined here as starch plus simple sugars (glucose, fructose, and sucrose). Gametophyte tissue samples and their corresponding media were analysed separately for NSC content following Hoch et al. (2002) and Bansal and Germino (2008). For soluble sugars, dried powder samples (4–5 mg ±0.1 mg) were boiled in water to extract sugars into solution. Media samples with sugar already in solution did not require boiling. Extracted tissue and media solutions were then enzymatically treated in stepwise fashion with invertase (to hydrolyse sucrose into glucose and fructose), phosphohexoisomerase (to convert fructose to glucose), and hexokinase (to convert glucose to 6-phosphogluconate; Sigma Diagnostics, St Louis, MO, USA). Oxidation of the simple sugars to 6-phosphogluconate resulted in an equimolar reduction of NAD to NADH. Increased absorbance of sample solutions at 340 nm (Synergy Microplate Reader; Biotek Instruments, Winooski, VT, USA) was directly proportional to simple sugar concentrations. Each 96-well microplate included calibration standards for each sugar. Total NSC (starch plus simple sugars) was determined with the same procedure described above except that starch in the original tissue samples was first degraded to glucose using fungal 6-glucoamylase (‘Clarase G-Plus’ from Aspergillus oryzae; Genecore International, Rochester, NY, USA) for 12–15 h, determined as the time when no further changes in absorbance occurred. Starch content was estimated as total NSCs minus total soluble sugars.

Stable-isotope analysis

Stable-isotope analysis of carbon in dry gametophyte tissue and culture media were completed using standard methods and a linear mixing model (Ehleringer et al., 1993; Lathja and Michener, 1994). Samples for carbon elemental concentration and stable isotope ratio were analysed at the Idaho State University Interdisciplinary Laboratory for Elemental and Isotopic Analysis (ILEIA) using a Costech ECS 4010 elemental analyser interfaced to a Thermo Delta V Advantage continuous flow isotope ratio mass spectrometer. All stable isotopic data are reported in standard delta notation (\( \delta^{13} \text{C} \)) relative to the Vienna PeeDee Belemnite (VPDB) reference standard. Analytical precision, calculated from analysis of standards distributed throughout each run, was ±0.2\%\text{PDB} or less and less than ±0.5\% of the sample value for 6\%\text{PDB}. Isotopic values are reported in the conventional \( \delta \)-notation \( \delta^{13} \text{C} = \left( \frac{R_{\text{sample}}}{R_{\text{standards}}} - 1 \right) \times 1000 \), where \( R = \frac{^{13} \text{C}}{^{12} \text{C}} \) relative to the international VPDB standard, expressed as per mil (%). \( \delta^{13} \text{C} \) was determined for gametophytes in all sugar treatments (\( \delta^{13} \text{C}_{\text{gametophyte-mixotrot}} \)) and controls (\( \delta^{13} \text{C}_{\text{gametophyte-autotrot}} \)), sugars added to the media (\( \delta^{13} \text{C}_{\text{sugar}} \)), and sugars remaining in media after 12 d of culture growth (n=2 samples, pooled from either two or three replicates, extracted by evaporating the medium). The proportion of gametophyte carbon derived from exogenous sugar (\( x \)) or photosynthesis (1–\( x \)) was determined using a mixing model, in which \( \delta^{13} \text{C}_{\text{gametophyte-mixotrot}} = x \delta^{13} \text{C}_{\text{sugar}} + (1-x) \delta^{13} \text{C}_{\text{gametophyte-autotrot}} \). This model provided a minimum estimate of the proportion of carbon from uptake, since plants grown in liquid media could also potentially reassorb a fraction of their own respired CO\(_2\). To estimate the upper limit of the proportion of carbon from uptake, the value of \( \delta^{13} \text{C}_{\text{gametophyte-autotrot}} \) in the mixing model was adjusted by assuming plants relied completely on reoxidation of their own respired CO\(_2\). In that case, the end member for gametophyte autotrophy was \( \delta^{13} \text{C}_{\text{gametophyte-autotrot}} \) \( + \delta^{13} \text{C}_{\text{air}} \).

Statistical analyses

One-way ANOVAs with Tukey–Kramer HSD tests (pre-set \( \alpha = 0.05 \) for HSD test; JMP, SAS Institute, Cary, NC, USA) for mean separations were used to determine: (i) the significance of differences in sugar depletion between the four sugar treatments or between single and blended hexose treatments, and (ii) effects of sugar treatments compared with no-sugar controls on dry mass gain, plant area, and carbon variables. To verify that the isotopically based estimates of the proportion of carbon taken up by gametophytes bathed in exogenous sugar was significantly different from zero (i.e. zero–complete autotrophy), we performed a one-way ANOVA and compared the maximum model output of the sugar treatments against the no-sugar treatment using Dunnett’s test (assigning the no-sugar treatment to the control). All assumptions of normality and heteroscedacity were met.

Results

C. richardii gametophytes depleted sugars from all of the bathing media (Fig. 1). Plants with a single hexose available tended to deplete more of the sugar initially added to
their media than plants in a blend of NSCs, but differences between treatments were not significant ($F = 2.78$, $P = 0.11$). Plants absorbed nearly an identical mass of fructose and glucose from their surroundings when either hexose was available (Fig. 1A). Plants growing in the FG hexose blend absorbed $\sim 30\%$ less sugar from their media than plants in either of the single hexose treatments (Fig. 1A). Although the mass of sucrose apparently depleted from the sucrose media (Fig. 1C) was $50\%$ greater than the depletions observed for either single hexose treatment (Fig. 1A), equimolar amounts of both fructose and glucose also appeared in the sucrose media. Each hexose reached a final concentration of $\sim 11 \text{ mM}$ after 12 d of plant growth in media that received sucrose (Fig. 1C). Thus, the actual net mass of sugar depleted by plants in the sucrose media was only $8\%$ (and not significantly) less than each of the single hexose treatments, assuming these hexoses were derived from sucrose hydrolysis by putative cwINVs. This assumption is partially supported by a lack of fructose or glucose emergence (0%) into the media of control plants grown with no sugar addition (data not shown).

The $\delta^{13}$C of autotrophic control plants grown without any exogenous sugars was $-29.6\%$, which indicated C3 photosynthesis (Fig. 2A). Gametophyte tissues grown in the presence of exogenous fructose, glucose, or sucrose with $\delta^{13}$C values near $-11\%$ (Fig. 2B) had $\delta^{13}$C values near $-18\%$, indicating significant sugar uptake ($F=787.3$, $P < 0.0001$; Fig. 2A). Based on the simple mixing model, at least $61\%$ of the carbon in mixotrophic gametophytes was derived from exogenous sugar and $39\%$ from photosynthesis. The proportion of tissue carbon from sugar uptake may have been as high as $75\%$ if plants used their own respired CO$_2$ for photosynthesis. The $\delta^{13}$C values of the sugar remaining in the media at the end of the experiment were the same as those of the source sugar used to prepare the media (Fig. 2B).

Fig. 1. Patterns of sugar depletion from liquid basal salts media containing either 60 mM fructose or glucose (A), 30 mM fructose plus 30 mM glucose (B), or 60 mM sucrose (C) after 12 d of gametophyte culture. Solid lines in each frame show the initial weight of each sugar added in each treatment. Bars show residual sugar concentrations remaining in media at the end of the growth period. Since the FG treatment included two hexoses, the total sugar added is double the amount shown by the solid line in (B). The dotted line in the sucrose plot shows the amount of fructose and glucose predicted if the disaccharide depleted from the media released hexoses due to native cwINV activity, and there was no uptake of either hexose. Data represent means±SD ($n=5$ cultures).

Fig. 2. $\delta^{13}$C in gametophyte dry mass (A) and exogenous sugar in media (B). $n=5$ cultures for gametophytes in the sugar media, and $n=2$ for control plants (no sugar, only autotrophy) and for sugars in their initial dry stock state or as extracted from media following 12 d of culture growth. In some instances, error bars are too small to be seen.
The availability of any exogenous sugar as either a single mono- or disaccharide, or as an equimolar blend of two monosaccharides significantly increased the mass and area of 12 d-old gametophytes by \( \geq 3 \)-fold relative to control plants grown without an exogenous sugar source \((P<0.001; \text{Fig. 3})\). Exogenous sugars of any kind also increased the greenness of the plants relative to the control by 27–36% \((P<0.001)\).

Total NSC content (soluble sugar plus starch) as a percentage of tissue dry mass was significantly increased in the presence of any of the exogenous sugar sources provided \((P<0.001; \text{Fig. 4A})\). Mixotrophic plant dry mass had \( \approx 3 \)- to 6-fold more soluble sugars and 5- to 7-fold more starch when compared with plants grown autotrophically \((\text{Fig. 4B})\). Plants grown in a blend of exogenous sugars accumulated a greater percentage of NSCs than plants grown in a single hexose alone \((\text{Fig. 4A, } P<0.001)\). The total percentage carbon content (NSCs plus structural carbon) in the dry mass of plants grown in fructose, glucose, or FG was also greater compared with autotrophic plants, although only by 3% \((F=4.58, P=0.01, \text{Fig. 4C})\). The total percentage carbon content of tissue from sucrose-grown plants was the same as the control plants \((\text{Fig. 4C})\). The increase in NSC due to sucrose uptake \((\text{Fig. 4A})\) thus corresponded with a proportional decrease in structural carbon, such that the total percentage carbon content in dry mass remained the same relative to control plants. In the remaining sugar treatments, increased NSC content corresponded with higher total carbon content \((\text{Fig. 4})\).

The patterns of carbon accumulation \((\text{Figs 2, 4})\) relative to patterns of carbon depletion from the media \((\text{Fig. 1})\) indicated that plants grown with a blend of NSCs available in their apoplast had higher average carbon-use efficiency than plants grown in a single hexose alone \((\text{for NSC, } F=11.8, P=0.003; \text{for structural carbon, } F=4.6, P=0.045; \text{Fig. 5A})\). Plants in the fructose or glucose treatments were almost only half as efficient \((\text{accumulation/depletion})\) as plants in the sucrose treatment \((F=5.18, P=0.035; \text{Fig. 5B})\). After accounting for the total carbon accumulated in plant dry mass, \( \geq 75\% \) of carbon depleted from the media in all of the sugar treatments ended up being lost from the cultures, more so from the single-hexose cultures \((86\% \text{ lost}; F=5.0, P=0.04; \text{Fig. 5C})\).

**Discussion**

**NSC uptake efficiency and sink strength in mixotrophic plants**

One of the most interesting results from the current work was the uncoupling of sink strength and carbon accumulation in green sink tissues, where sink strength is defined as the plant’s net depletion of NSCs from the exogenous source pool. Bathing plants simultaneously in a blend of fructose and glucose improved sink efficiency, i.e. the ratio of total carbon accumulation/total carbon depleted from the media \((\text{Fig. 5A, B})\). By the end of the 12-d experiment, the sucrose media contained the disaccharide plus \( \approx 11 \) mM concentrations of fructose and glucose \((\text{Fig. 1C})\). We attribute the appearance of the hexoses in the sucrose treatment to the putative hydrolysis of sucrose by cwINVs that released fructose and glucose into the media, as seen in other in vitro studies \((\text{Kretzschmar } \text{et al., 2007})\).
Consequently, the sucrose media ultimately developed an excess of three different carbohydrate sources potentially available for uptake from a plant’s apoplast. Since fructose and glucose were clearly transported, it was impossible to determine whether any of the disaccharide was also directly transported or if it was strictly hydrolysed into the constituent monosaccharides prior to uptake. Nevertheless, plants grown with a single hexose present in the media were only 57% as efficient as plants originally provided with sucrose, and the response was essentially identical regardless of which hexose was available (Fig. 5B). Plants grown in a blend of fructose and glucose were 87% as efficient as sucrose-grown gametophytes, although this difference was not significant (Fig. 5B). Higher carbon-use efficiency in the presence of an equal mix of exogenous fructose and glucose might be expected if plants are normally adapted for uptake of equimolar concentrations of these two hexoses after sucrose hydrolysis in the apoplast. It is also possible that sucrose had some specific additional effects on plant metabolism via sucrose signalling (Pollack and Farrar, 1996), since plants grown in exogenous sucrose tended to have somewhat higher carbon-use efficiency than plants in FG (Fig. 5B). These effects would be absent in plants that fed directly on a blend of fructose and glucose.

One potential explanation for the differences in sink efficiency observed among mixotrophic plants is that sugar uptake enhanced photosynthesis and shifted the balance of carbon assimilation away from heterotrophy and towards autotrophy. The darker green plants associated with sugar treatments was consistent with an increase in chlorophyll content and potentially greater photoassimilation by gametophytes grown in sugar. However, the discrimination against $^{13}$C by mixotrophic plants was nearly identical in all sugar treatments (Fig. 2A). The presence of any exogenous sugar may indeed enhance photosynthesis in gametophytes compared with control plants grown without sugar, but the stable isotope data also suggest similar photosynthetic carbon uptake for plants that were importing different types of sugar.

A more likely explanation for the variation in sink efficiency between sugar treatments is that the uptake of different sugar combinations had different effects on respiration. Photosynthetic plants can lose $\geq 50\%$ of their assimilated carbon to dark respiration (Farrar, 1985). Putative respiratory loss of carbon in fully heterotrophic cultures can be estimated as the amount of carbon lost from the medium that is not incorporated into the final tissue dry mass. The actual rate of respiratory loss from mixotrophic

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**Fig. 4.** The effect of exogenous sugar treatments on total NSC content (A, B) and total carbon content (C) of 12-d-old gametophyte tissue as a percentage of total plant dry mass. There was a significant effect of sugar treatment on total NSC content ($P<0.001$), and plants grown in the presence of either sucrose or FG both had higher NSC content than plants grown in either of the two hexoses alone ($P<0.029$). The majority (79–87%) of NSC content of plants grown in exogenous sugars was starch. Compared with no-sugar control plants, the elevated NSC content was associated with higher total carbon content in all sugar treatments except sucrose. All means are based on $n=5$ cultures, except for total carbon in the no-sugar treatment ($n=2$). Means with the same letter within (A) and (C) were not significantly different. Error bars show one standard deviation.
Gametophytes was slightly greater than this estimate because plants bathed in exogenous sugars also assimilated up to 39% of their carbon by photosynthesis (Fig. 2A). An average of 86% of the carbon depleted from both the fructose and glucose media was unaccounted for in the final tissue dry mass, whereas plants grown in the sucrose and FG solutions lost an average of 75–78% of the carbon depleted from their media (Fig. 5C). The progressive decline in respiratory carbon loss among sugar treatments (fructose > glucose > FG > sucrose) was proportional to the progressive increase seen in carbon-use efficiency (fructose > glucose > FG > sucrose) (Fig. 5B).

Improved sink efficiency resulting from the simultaneous uptake of fructose and glucose while sink cells also conduct photosynthesis may reduce the average energy costs of cellular metabolism. Mixotrophic plants importing apoplastic fructose, glucose, and potentially sucrose into the cell cytosol while fixing additional carbon in chloroplasts should reduce the need to interconvert and transport carbohydrates at levels normally required to maintain stoichiometry in carbon metabolic pathways of autotrophic cells. This could account for the inverse relationship observed here between putative respiratory demand and the number of sugar types available for uptake during mixotrophy. Flux balance analysis of metabolism in the photosynthetic prokaryote *Synechocystis* also indicated that mixotrophic growth with a single hexose (glucose) under optimal lighting is more efficient than either heterotrophy or autotrophy (Shastri and Morgan, 2005). The results from *C. richardii* gametophytes suggest that biomass yield efficiency in other single-hexose, mixotrophic culture systems can potentially be improved if cells that are capable of importing a diversity of exogenous metabolites are fed a mixture of sugars simultaneously.

The overall high levels of carbon loss by respiration observed in mixotrophic gametophytes were striking and initially suggested futile cycling of carbohydrate uptake followed by respiratory loss. Plants had a large surplus of carbon available throughout their development that they could not possibly use in the allotted time (i.e. they were sink limited). Other studies in seed plants show that the accumulation of NSCs can subsequently limit photosynthesis by feedback inhibition (Paul and Foyer, 2001; Kasai, 2008), so it was reasonable to expect that mixotrophic gametophytes might cope with elevated NSC levels by conducting very little photosynthesis. However, the isotopic evidence suggested that 25–39% of tissue carbon was still derived from photosynthesis even when plants were fed exogenous sugars. The actual contribution of photosynthesis depended on how often plants reassimilated respired CO$_2$ with $\delta^{13}$C values more negative than CO$_2$ in air. Gametophytes were grown in very low light and 75–86% of the carbon depleted from the media was not retained in the final plant dry matter (Fig. 5C). If the exogenous sugar pool entering the cytosol was preferentially used as a respiratory substrate, the photoassimilated carbon pool could accumulate to significant levels even if the absolute rates of photoassimilation were low. Future studies that include direct measurement of respiration during mixotrophic plant growth in the presence of isotopic tracers would show whether plants selectively release the absorbed exogenous-sugar pool as CO$_2$.

In sink-limited species, increasing the non-phosphorylating alternative oxidative pathway in non-green sink tissues is hypothesized to alleviate the down-regulation of photosynthesis in source tissues caused by a carbon surplus (Lambers, 1982; Gandin *et al.*, 2009). The alternative oxidative pathway can respire excess carbon from sink tissues without generating harmful reactive oxygen species, and this allows sinks to continue to import additional carbohydrates. The response of gametophytes in this study was consistent with this hypothesis except that photosynthesis and sink limitation occur within the same green plants rather than in spatially segregated source and sink organs connected by phloem. Gametophytes grown in...
exogenous sugars became greener and obtained up to 39\% of their carbon from photosynthesis, while the absolute rates of carbon loss from the cultures due to putative respiration were very high. The $\delta^{13}$C values of plant dry mass were also all very similar, regardless of the type of exogenous sugar available for uptake and regardless of differences in respiratory loss between sugar treatments. This suggests that the alternative respiratory pathway may do more than simply release excess carbon when green sinks become sink limited. Avoiding the down-regulation of photosynthesis by up-regulating respiration may be a homeostatic adaptation that allows green sinks to sustain a certain mixotrophic set point (i.e. a stable ratio of sugar imported from external source tissues and sugar from in situ photosynthesis).

In angiosperms, the presence of exogenous sugars commonly results in a decrease in chlorophyll content (Posner, 1970; Krapp et al., 1991; Stevenson and Harrington, 2009). However, exogenous sugar can increase chlorophyll content under some culture conditions (To et al., 2003). An increase in chlorophyll due to exogenous sucrose treatments is also a feature of some in vitro tissue culture studies (George et al., 2007) and links between sugar uptake and photosynthesis rates have also been demonstrated (Van Le et al., 2001; Fuentes et al., 2005a). Interestingly, intermediate combinations of light flux and sucrose levels can foster a beneficial balance between increased photoassimilation and sucrose heterotrophy (i.e. mixotrophy) that results in enhanced in vitro growth and explant survival in Nicotiana, coconut, and tomato (Tichá et al., 1998; Van Le et al., 2001; Fuentes et al., 2005b). Evolutionary optimization of this mixotrophic behaviour may also be a relevant adaptation in seed plant sinks in planta (e.g. developing fruits) specifically where apoplastic phloem unloading occurs in immature sink tissues that remain green and conduct their own ‘sink’ photosynthesis over an extended period of time. Smillie et al. (1999) have previously noted that the upper hemisphere of immature tomato fruits has high chlorophyll content and pericarp photosynthesis may be a key factor determining the final ratio of sugar to acid in mature fruit. This may be especially important as continued plant growth results in self-shading of developing fruit.

Conclusions

Ferns and other non-seed plants form a monophyletic group that is sister to the seed plants (Pryer et al., 2001). In spite of a long period of independent evolution, the results to date from _C. richardii_ and other non-seed plants (Renault et al., 1992; Johnson et al., 2006) support the view that basic mechanisms of apoplastic sugar transport are relatively conserved across all land plants. _C. richardii_ consequentially presents a very tractable model to gain a broader evolutionary view of the interrelated processes of photoassimilation, NSC transport, and respiratory metabolism in a life cycle that includes plants with and without phloem.

To our knowledge, this study is the first to show that gametophytes in this semi-aquatic model fern system conduct C3 photosynthesis. It also provides the first confirmation that at least one member of the sugar transporter gene family in this non-seed plant transports fructose at significant rates. Klepek et al. (2010) recently showed that two polyol/monosaccharide transporters in _A. thaliana_ allow high-capacity fructose uptake by developing pollen. Pollen also hydrolyses sucrose with an extracellular invertase (Ylstra et al., 1998). Fructose feeding by mixotrophic _C. richardii_ gametophytes suggests this trait may be primitive and widespread among land plants.

Evidence is accumulating that mixotrophy is a primitive trait with potentially important ecophysiological consequences for early land plants (Graham et al., 2010). Growing _C. richardii_ gametophytes under mixotrophic conditions revealed that the highest carbon accumulation efficiency was specifically due to the availability of a blend of simple sugars to these green plants, not just a surplus of any apoplastic carbohydrate. This fact was made evident because we were able to readily quantify the increase in NSC and structural carbon within gametophytes not only as a function of total tissue dry mass, but also as a correlated function of sugar uptake from the surrounding medium. In this study system, it now appears likely that apoplastic sugar feeding by gametophytes shares at least some functional similarities previously attributed to post-sieve element transport across the apoplast into sinks (LaLonde et al., 2003). If so, the study of mixotrophic carbon accumulation efficiency merits more careful attention in economically important vascular plants, where fructose and glucose released by cwINVs modulate apparent sink strength in green sink tissues.

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