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Increasing the carbohydrate storage capacity of plants by engineering a glycogen-like polymer pool in the cytosol☆

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ABSTRACT

Global demand for higher crop yields and for more efficient utilization of agricultural products will grow over the next decades. Here, we present a new concept for boosting the carbohydrate content of plants, by channeling photosynthetically fixed carbon into a newly engineered glucose polymer pool. We transiently expressed the starch/glycogen synthases from either *Saccharomyces cerevisiae* or *Cyanidioschyzon merolae*, together with the starch branching enzyme from *C. merolae*, in the cytosol of *Nicotiana benthamiana* leaves. This effectively built a UDP-glucose-dependent glycogen biosynthesis pathway. Glycogen synthesis was observed with Transmission Electron Microscopy, and the polymer structure was further analyzed. Within three days of enzyme expression, glycogen content of the leaf was 5–10 times higher than the starch levels of the control. Further, the leaves produced less starch and sucrose, which are normally the carbohydrate end-products of photosynthesis. We conclude that after enzyme expression, the newly fixed carbohydrates were routed into the new glycogen sink and trapped. Our approach allows carbohydrates to be efficiently stored in a new subcellular compartment, thus increasing the value of vegetative crop tissues for biofuel production or animal feed. The method also opens new potential for increasing the sink strength of heterotrophic tissues.

1. Introduction

Carbohydrates produced by plants are a staple component of human and animal diets and serve as essential ingredients in many manufacturing processes, including biofuel production. Starch is one of the most commercially important carbohydrates and is composed of glucose polymers. Heterotrophic storage organs such as seeds, roots and tubers can store up to 80% of their dry weight as starch. Photosynthetic tissues also have a high capacity to synthesize starch. In leaves, a portion of the carbon fixed in the Calvin cycle is stored as starch, which is used to support metabolism during the night (Zeeman et al., 2010). The starch content of leaves is generally lower than that of storage organs due to this diurnal turnover.

Increasing the total carbohydrate yield is a major goal in biotechnology agriculture. Several approaches have been explored with varied success, and have mainly focused on starch storage organs (Smith, 2008). Strategies included methods to increase photosynthesis (Lin et al., 2014; Long et al., 2015), manipulate sink-source relations (Borras et al., 2004), alter carbon partitioning, or to block transient starch breakdown (Weise et al., 2012). Green tissues have lower commercial value than storage tissues as they contain lower amounts of readily accessible carbohydrates and higher water content (up to 90%). As a result, their potential for use in industrial applications is often overlooked (Ohlrogge et al., 2009), and few attempts have been made to increase the carbohydrate content of these tissues. Increased amounts of readily accessible carbohydrate (such as starch or glycogen), rather than recalcitrant lignocellulosic material, could be beneficial for the second-generation biofuel industry. This would be a major step towards sustainable energy, encouraging the utilization of otherwise unused plant material, reducing waste and avoiding competition with food/feed stock (Naik et al., 2010). Additionally, higher levels of readily-digestible carbohydrates in vegetative tissues improve forage and silage by increasing calorific value (Kingston-Smith et al., 2013).

Therefore, we conceived a strategy to increase the carbohydrate content of green tissues in plants, utilizing enzymes from the cytosolic starch/glycogen synthesis pathway from other organisms (red algae and yeast), and taking advantage of the storage potential of the extraplastidial space in plant cells. Living organisms have developed different approaches to store carbohydrates. Bacteria and non-photosynthetic eukaryotes store glycogen in the cytosol. This soluble glucose polymer consists of relatively short, linear α−1,4-linked glucose chains, linked via frequent α−1,6-linkages (branch points). Red algae synthe-

☆ Engineering cytosolic glycogen in plants.

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size either glycogen-like or starch-like (floridian starch) glucose polymers in the cytosol (Shimonaga et al., 2008). By contrast, plants and green algae produce insoluble semi-crystalline starch granules in plastids, and the granules contain two different glucose polymers (amylopectin and amylose). Amylopectin accounts for most of the granule mass (around 60–90%), and is also a branched polymer, but it has longer chains and fewer branch points than glycogen. The structure of amylopectin allows linear chain segments to align and form double helices, which pack into semi-crystalline lamellae (Buleon et al., 1998; Zeeman et al., 2007). Glycogen and amylopectin synthesis follow a similar basic principle. Both require a primer to initiate the synthesis of the polymer. For glycogen synthesis, this primer is a self-glycosylating protein (Lomako et al., 1988; Ugalde et al., 2003). For amylopectin the exact priming mechanism is unknown. Glycogen synthases (GS's) and starch synthases (SS's) are related glucosyltransferases, which use nucleotide-activated glucose donors to extend pre-existing chains. GS's in animals and fungi and SS's in red algae (*Rhodophyta*) use UDP-glucose, whereas SS's in plants and green algae and GS's in prokaryotes use ADP-glucose (Ball et al., 2011). Branching enzymes (BE's) cleave off an existing α−1,4-linked chain, and transfers the cut segment to another chain, creating a new α−1,6 branch point.

Here, we created a new glycogen-like polymer pool in the cytosol of leaf cells, utilizing GS/SS isoforms that can exploit the cytosolic UDP-glucose pool. The polymer avoided degradation by the chloroplastic starch-degrading enzymes, and acted as a strong carbohydrate sink. This approach greatly increased the total non-structural carbohydrate content of leaves, resulting in glucose polymer levels ten times higher than those typically found in leaves.

2. Materials and Methods

2.1. Cloning and constructs

The coding sequence of *Cyanidioschyzon merolae* alpha-1,4-glucan branching enzyme (named *Cm*BE; XP_005536101.1) was codon optimized for expression in plants (Supplemental file 1), and cloned into the Gateway-compatible entry vector, pDONR221 (ThermoFisher Scientific). Sequences encoding *C. merolae* starch/glycogen synthase (named *Cm*GS; XM_005537117.1) and *Saccharomyces cerevisiae* GSY2 (named *Sc*GSY; NM_001182145.1) were amplified from genomic DNA, and cloned into pDONR221. The Ubiquitin 10 promoter (pUBQ10) was cloned into the entry vector, pDONR221 P4-P1R (ThermoFisher Scientific). Sequences encoding GFP and mCherry fluorescent proteins were cloned into the entry vector pDONR221 P2R-P3 (ThermoFisher Scientific). To generate the expression plasmids, the appropriate entry clones were recombined into the three-fragment Multisite Gateway-compatible destination vector pK7m34GW for *Cm*BE, and pB7m34GW for *Cm*GS and *Sc*GSY. All primers used for the cloning are listed in Supplemental Table 1.

2.2. Plant transformation and growth conditions

Expression vectors were transformed into *Agrobacterium tumefaciens* (strain GV3101), and cultures were grown at 30 °C in LB medium (Chemie Brunschwig) supplemented with the appropriate antibiotics for 16 h. Cells were pelleted at 5000 g for 15 min and washed once with infiltration media containing 50 mM MES (Sigma-Aldrich) pH 5.7, 2 mM sodium dihydrogen phosphate dihydrate (Sigma-Aldrich), 0.5% glucose monohydrate (Sigma-Aldrich), 100 µM acetosyringone (3′,5′-dimethoxy-4′-hydroxyacetophenone) (Sigma-Aldrich). After washing, cells were resuspended in infiltration media to an OD_{600} of 1. For the co-infiltration of multiple constructs, the appropriate resuspended cultures were mixed at equal volumes. The P19 silencing suppressor was added to all combinations by co-infiltrating *A. tumefaciens* carrying a 35 S:P19 construct (Marillonnet et al., 2005).

Nicotiana benthamiana plants were grown for 5 weeks in growth chambers (CLF Plant Climatics) under a 12-h-light/12-h-dark regime at 20 °C, 65% relative humidity and light intensity 150 µmol quanta m^{-2} s^{-1}. The agrobacterium suspensions were infiltrated into leaves using a 1 mL syringe. After infiltration, plants were returned to the growth chamber.

2.3. Confocal and electron microscopy

Confocal images were acquired on a Zeiss LSM780 confocal microscope (Carl Zeiss), with a 40× water-immersion lens with a 1.1 numerical aperture. GFP fluorescence was excited using an argon laser at 488 nm wavelength and emitted light was captured at 536 nm. mCherry fluorescence was excited using an argon laser at 594 nm and emitted light was captured at 648 nm. Chlorophyll autofluorescence was excited with an argon laser at 633 nm and emitted light was monitored at 684 nm. Images were processed using ImageJ software (http://rsbweb.nih.gov/ij/).

For transmission electron microscopy, *N. benthamiana* leaves were harvested 3 days after infiltration. Small leaf segments were fixed in 2% (v/v) glutaraldehyde (Sigma-Aldrich) in 0.05 M sodium cacodylate buffer, pH 7.4, (Sigma-Aldrich, C4945) for 6 h under vacuum at 20 °C. After washing three times with 0.1 M sodium cacodylate buffer, pH 7.4, the samples were incubated for 16 h in 1% (w/v) osmium tetroxide (Chemie Brunschwig, 0223A-5) in 0.1 M sodium cacodylate buffer, pH 7.4, at 4 °C. Samples were then washed three times in cold 0.1 M sodium cacodylate buffer, pH 7.4, and once with water. The samples were dehydrated in a series of aqueous ethanol solutions from 50% (v/v) to 100% ethanol and once with 100% acetone (Thommen-Furler AG). The 100% ethanol and 100% acetone was dehydrated over a 3 Å molecular sieve (Carl Roth, 8487.1) prior to use. After dehydration, the leaf sections were incubated for 2 h in 25% (v/v) EPON epoxy resin (diluted with 100% acetone), 2 h in 50% (v/v) EPON epoxy resin, 16 h in 75% (v/v) EPON epoxy resin, and 7 h in 100% EPON epoxy resin (EPON epoxy resin consists of: 14.5 g “Epoxy embedding medium” [Sigma-Aldrich, 45345]), 10.5 g “Epoxy embedding medium, hardener MNA” [Sigma-Aldrich, 45347], 5.0 g “Epoxy embedding medium, hardener DDSA” [Sigma-Aldrich, 45346], 0.54 g “Epoxy embedding medium, accelerator DMP30″ (Sigma-Aldrich, 45348]). Embedding was completed in fresh 100% EPON epoxy resin by incubating at 70 °C for 48 h. Ultrathin sections were cut with a diamond knife, collected on copper grids, and stained with 2% (w/v) uranyl acetate (Sigma-Aldrich, 73943). After three washes with water, sections were stained in Reynold's lead citrate (Reynolds, 1963). Stained sections were washed three times with water and examined using a FEI Morgagni 268 transmission electron microscope.

2.4. Lugol staining

Leaves were harvested and decolorized in 80% (v/v) ethanol at 60 °C. Excess ethanol was removed, before staining with Lugol solution (Sigma-Aldrich, 32922). After staining, leaves were washed in water.

2.5. Analysis of starch/glycogen synthase and branching enzyme activity by native PAGE

Infiltrated leaves were harvested and frozen in liquid N_2. Proteins were extracted in their native state in extraction medium (100 mM MOPS, pH 7.2, [Sigma-Aldrich], 1 mM EDTA [Sigma-Aldrich], 1 mM DTT [Sigma-Aldrich,], 10% [v/v] glycerol, and Complete protease inhibitor cocktail [Roche, 05,056489001]) using a mixer mill (RETSCH).

Proteins (20 µg) were separated on a 7.5% native polyacrylamide gel containing 0.03% (for BE activity) or 0.3% (for synthase activity) oyster glycogen (Sigma-Aldrich, G8751), 375 mM Tris-HCl pH 8.8

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