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Research article

Starch accumulation is associated with active growth in *A. tequilana*

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ABSTRACT

Transcriptome analysis of different tissues and developmental stages of *A. tequilana* plants led to the identification of full length cDNAs and the corresponding amino acid sequences for enzymes involved in starch metabolism in this species. Comparison with sequences from other species confirmed the identities of putative *A. tequilana* starch metabolism genes and uncovered differences in the evolutionary patterns of these genes between gramineous and non-gramineous monocotyledons. *In silico* expression patterns showed high levels of expression of starch metabolism genes in shoot apical meristem tissue and histological studies showed the presence of starch in leaf primordia surrounding the shoot apical meristem and in the primary thickening meristem of the stem. Starch was also found to accumulate significantly in developing floral organs and immature embryos. Low levels of starch were observed overall in leaf tissue with the exception of stomatal guard cells where starch was abundant. In root tissue, starch was only observed in statoliths at the root tip. *A. tequilana* starch grains were found to be small in comparison to other species and have an almost spherical form. The data for gene expression and histological localization are consistent with a role for starch as a transient carbohydrate store for actively growing tissues in *A. tequilana*.

1. Introduction

Agave species have been exploited in Mexico since the pre-hispanic era (Gentry, 1982; García-Mendoza, 1992, 2000; García, 2011) and are currently of commercial importance for the preparation of alcoholic beverages such as tequila and mescal (https://www.crt.org.mx/, http://www.crm.org.mx/). Most *Agave* species are perennial and monocarpic with life cycles ranging between 5 and 50 years and are well adapted to dry marginal terrain having shallow roots, leaves organized in rosette formation, thick cuticles and crassulacean acid metabolism (CAM) (Gentry, 1982). *Agaves* are also included in the 15% (Hendry, 1987, 1993; Brocklebank and Hendry, 1989) of angiosperms that store carbohydrates in the form of fructans which are predominantly stored in the expanded stem structure but are also found in almost all organs including leaves, roots and floral tissue (Avila de Dios et al., 2015; Mancilla-Margalli and Lopez, 2006). Low molecular weight fructans can be mobilized from leaves through the phloem (Wang and Nobel, 1998) and in addition to carbohydrate storage fructan polymers are thought to have roles in stress tolerance, regulation of osmosis and signaling (Van den Ende and Valluru, 2009; Van den Ende et al., 2004; Van den Ende, 2013; Van den Ende, 2014).

In contrast to *Agave* species, the majority of angiosperms store carbohydrates both transiently and long-term in the form of starch (Zeeman et al., 2010). Transient starch storage occurs in leaves and stems where levels can be modulated in accordance with the needs of the plant, whereas long-term storage is associated with specific organs such as seeds, roots or tubers (Zeeman et al., 2010). Within the monocotyledons starch metabolism has evolved differently within distinct taxonomic groups (Comparot-Moss and Denyer, 2009). The Commelinid clade which houses gramineous species such as cereals, sedges and banana species is characterized by species that produce seeds with starch-rich endosperm tissue. Other monocotyledonous clades however, including the Asparagales (containing the *Agave* genus) and Lilliales do not significantly accumulate starch in seed endosperm. ADPglucose is an essential starch precursor synthesized in the chloroplasts of all angiosperms. Gramineous species however, are further distinguished by the ability to also synthesize this molecule in the cytosol due to the presence of an extra AGPase enzyme in this compartment, indicating fundamental differences in the evolution of starch metabolism encoding genes in gramineous and non-gramineous

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monocotyledons (Comparot-Moss and Denyer, 2009; James et al., 2003).

Several monocotyledonous species synthesize both starch and fructans and starch localization and metabolism has been studied in some fructan producing monocotyledons including grass species such as wheat (*T. aestivum*) (Scofield et al., 2009) and non-gramineous species such as onion (*A. cepa*) and Asparagus (Schnabl and Ziegler, 1977; Zhang et al., 2016) (Ernst et al., 1998, 2003; Ernst and Krug, 1998) where some evidence for both spatial and temporal separation of starch and fructan metabolism has been uncovered.

Agave species in common with *Allium* species are non-gramineous monocotyledons found in the Asparagales clade. Although these species do not accumulate starch in endosperm tissue or other specialized organs, it could be expected that starch would be transiently synthesized in leaves as a consequence of photosynthesis in these species. However Christopher and Holtum (1996) reported that starch is not stored in *Agave guadalajarana* leaves and only low levels of starch are detected in *A. tequilana* stems (Mancilla-Margalli and López, 2006). Additionally in *A. cepa* (a species relatively closely related to the *Agave* genus), the absence of starch in stomatal guard cells has been reported (Schnabl and Ziegler, 1977; Schnabl et al., 1978; Schnabl, 1980).

The localization and role of starch metabolism in *Agave* species is therefore unclear and since there is a growing interest in the use of Agaves as renewable bioenergy sources (Yang et al., 2015; Liu et al., 2015; Cushman et al., 2015; Davis et al., 2011) based on their capacity for biomass production (a trait linked directly to photosynthesis and carbohydrate metabolism) and the possibility to exploit marginal land, a fundamental knowledge of the roles of both fructan and starch metabolism in these species and how they interact is essential. This report begins to address this problem by characterizing cDNA sequences and determining *in silico* expression patterns in *A. tequilana* for genes encoding key enzymes in starch metabolism and describing the pattern of starch localization and the size and shape of the starch grains observed based on the histological analysis of different organs and tissues of this species. The results confirm a distinct pattern of evolution for starch metabolism genes between gramineous and non-gramineous monocotyledonous species and patterns of starch localization in combination with *in silico* expression data lead us to suggest that starch rather than fructans is the primary carbohydrate source exploited in actively growing tissues of *Agave* species.

2. Materials and methods

2.1. Plant material

For vegetative tissues, samples for histochemical and microscopical analysis were obtained from at least three, two-year old greenhouse grown *A. tequilana* plants (Supplementary Fig. 1a). For each plant, samples were obtained from: the leaf base and middle section of 2 individual leaves (both adaxial and abaxial areas), the center of the stem, the center and tip of the root, the shoot apical meristem (SAM) and rhizome tissue. Additionally adaxial and abaxial epidermis from middle-leaf tissue (Supplementary Fig. 1b), were analyzed. Depending on the tissue both transversal and longitudinal sections were obtained and were either fixed or analyzed as fresh tissue. Samples for analysis of floral structures (umbel, inflorescence, immature bud, seeds) were obtained from mature plants with an inflorescence showing floral buds (Supplementary Figs. 1c and d).

2.2. Multiphoton microscopy and rhodamine B staining

Samples were imaged on a multiphoton microscopy system (LSM 880NLO, Zeiss, Germany) equipped with a Ti: Sapphire laser (Chameleon vision II, COHERENT, Scotland) capable of tuning in ranges from 690 to 1060 nm. *A. tequilana* tissues were observed with a short working distance objective 20X/0.5, NA ∞-0.17, Zeiss Plan NEOFLUAR and starch localization was carried out with an immersion objective 40X/1.3, NA ∞-0.17, Zeiss Plan NEOFLUAR. The rhodamine B spectrum was obtained from the data-base of Zeiss microscope dyes. The spectrum was divided into two ranges, small window detection from 547 to 602 nm for visualization of cells walls and cuticle (in green) and large window detection from 600 to 735 nm for visualization of starch grains (in magenta) (Supplementary Fig. 2). All micrographs were captured in .CZI format at 1131x1131 pixels and RGB colour.

For the emission spectrum analysis of starch, 1 mg of potato starch grains (Merck, Germany) was mixed with 100 μL of ethanol/rhodamine solution (Hycel, Mexico) at 0.01% and incubated for 5 min, at room temperature. Subsequently the sample was washed three times with deionized water. The *A. tequilana* tissues (leaf primordia, expanded leaf and root) were dissected in 2 mm thick sections and dehydrated in a series of ethanol solutions (from 10 to 50%) for 30 min at room temperature. Subsequently, samples were incubated in an ethanol–glycerine-water solution, in ratios of 1:1:2, 1:1:1 for 12 h, in the final step absolute ethanol and glycerine were added in equal proportions (1:1) and samples were incubated for 24 h. Tissues were washed three times with a 50% ethanol solution and incubated in 1 mL of ethanol-acetone rhodamine solution at 0.01% for 10 min at room temperature. The staining process was completed by washing samples and leaving them for 72 h in deionized water at 4 °C. The tissues were mounted on glass slides, covered with high performance Zeiss cover glasses (D = 0.17 mm ± 0.005 mm refractive index = 1.5255 ± 0.0015, Abbe number = 56 ± 2) and observed in a multiphoton microscope.

2.3. Lugol staining

Apical, middle and basal *Agave tequilana* leaf tissue was dissected and incubated in graded solutions of sucrose (10–30%) dissolved in phosphate buffer at 0.16 M, pH 7.4 for 2 h at 4 °C. Subsequently, samples were embedded in LEICA tissue freezing medium (Leica Biosystems, United Kingdom) and frozen at −35 °C for 10 min. Tissues were cut in sections of 30 μm with high profile microtome 818 blades (Leica Microsystems, Germany). For starch detection, samples were incubated with lugol solution (1% in 50% ethanol) for 15 min and washed for 30 min.

Freehand sections of around 5 mm were obtained for umbel, floral meristem, root, stem and leaf tissues. Samples were incubated with lugol solution (1% in 50% ethanol) for 15 min, washed for 30 min and mounted on glass slides. Samples were observed in a Keyence VHX5000 digital microscope (Keyence, Japan), at 200X amplification (VH-Z20R, Keyence, Japan) with LED illumination.

2.4. Paraffin sections

The protocol was modified from (Sánchez-Segura et al., 2015). Root, central stem, basal and middle leaf tissues of *A. tequilana,* as well as positive controls rice (grain) and potato (tuber) were fixed during 24 h in 4% paraformaldehyde (Electron Microscopy Sciences, USA) dissolved in phosphate buffer, pH7.2 for 12 h, after which they were washed three times with phosphate buffer during 30 min at room temperature. Subsequently, the samples were dehydrated in a graded ethanol series (from 10% to 100%) and cleared with xylol and infiltrated with xylol-paraplast (2:1, 1:1, 1:2 and pure paraplast). Residual xylol was evaporated during 24 h at 60 °C, before embedding in pure Paraplast (McCormick Scientific, USA). Blocks were cut to 10 μm in width in a rotary retracting microtome (Liljeholmen, Kema and Bryggerierna (LKB), Sweden). The paraffin ribbons were de-waxed and cleaned in xylol, the rehydration of the sections was carried out in ethanol solutions (from 100% to 50%) and samples were stained with Lugol solution as described above and mounted in Entellan resin (Merck, Germany). The sections were examined under a light microscope (Olympus, BX50-BF, Japan) with an Infinity3 camera 1.4 MP (Lumenera, Canada) and ImagePro Premier 9.1 (Media Cybernetics,