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Differential fructan accumulation and expression of fructan biosynthesis, invertase and defense genes is induced in *Agave tequilana* plantlets by sucrose or stress-related elicitors☆

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ABSTRACT

The effect of short-term treatments with non-toxic concentrations of chemical elicitors of carbon mobilization and/or defense responses on fructan accumulation and complexity was analyzed in *Agave tequilana* plantlets. These included sucrose (ExSuc), salicylic acid (SA), and methyl jasmonate (MeJA). Methyl viologen (MV), an oxidative-stress elicitor, was also tested. Stems of ExSuc- and SA-treated agaves accumulated fructan with contrasting degree of polymerization (DP), being higher (DP12) in the ExSuc treatment. The difference agreed with 1-SST, 6G-FFT and 6-fructan exohydrolase (FEH) gene expression patterns. Thus, a strong, 6G-FFT expression detected 6 days after treatment (DAT), coupled to a systematic repression of FEH genes occurred in Ex-Suc treated agaves. Conversely, SA treatment induced maximum 6G-FFT expression and a transitory induction of FEH genes 2 DAT. MV also induced an accumulation of low-DP fructans 6 DAT. Additionally, it stimulated the highest fructan accumulation in leaves. Contrariwise, MeJA led to a depletion of soluble non-structural carbohydrates (NSCs) and fructan, particularly in leaves. An inverse relationship between high invertase and FEH gene expression levels and minimal NSCs and fructan reserves was observed in response to MeJA. Low DP fructan accumulation by MV could not be attributed to a measurable oxidative stress. Still, high antioxidant enzyme activity, indirectly manifesting oxidative stress, coincided with fructan accumulation in MV-treated agaves. High invertase and FEH expression levels induced by MeJA in leaves, and to a lesser degree by SA and MV, coincided with transcript accumulation of defense-related genes, and were, to a certain extent, in accordance with the "sweet immunity" concept, linking sugar and defense signaling.

1. Introduction

Agaves (*Agave* spp.) are perennial monocarpic plants that fix CO_2 via the Crassulacean Acid Metabolism pathway, which is predominantly employed by plants adapted to survive in arid environments (Pimienta-Barrios et al., 2005). Agaves are native of the American continent. Mexico, in particular, possesses the highest biodiversity of Agave plants, being indigenous to 75% of the approximately 200 known species (García-Mendoza and Galván, 1995). Agaves have been used for generations for the production of fructose-rich foods and beverages, distilled spirits and fibers (Mancilla-Margalli and López, 2006; García-Mendoza, 2007).

Constrained by their sessile lifestyle, plants have been compelled to develop a diversity of distinct mechanisms in order to adapt to unfavorable environmental conditions (Van den Ende and El-Esawe, 2014). A singular adaptive strategy adopted by several plant species to tolerate several abiotic stresses, including those caused by high temperatures, freezing, water-deficit and/or excessive salinity, involves the accumulation of fructan, most likely in the cell vacuole, although their transfer to the apoplast under stress conditions has also been proposed (Livingston and Henson, 1998; Valluru et al., 2008). Fructans are water-soluble plant carbohydrates (CHOs); chemically, they are β-(2, 1) and/or β-(2, 6)-linked fructose polymers anchored to a glucose residue. They have various levels of organization in terms of structure and size, which depend on diverse taxonomic, physiological and environmental factors.

Abbreviations: 1-SST, sucrose: sucrose 1-fructosyl transferase; 1-FFT, fructan: fructan 1-fructosyltransferase; 6G-FFT, fructan: fructan 6-G fructosyltransferase; DP, degree of polymerization; FEH, fructan exohydrolase; CWI, cell-wall invertases; VI, vacuolar invertases; SA, salicylic acid; MeJA, methyl jasmonate; MV, methyl viologen; FSG, Fructan Synthetic Genes; NSC, Non-structural carbohydrates.

☆ Main conclusion: Differential fructan accumulation/complexity was induced by diverse elicitors in *Agave*. Changes in fructan- and sucrose-related genes were involved. The results lent support to the "sweet immunity" and fructan as antioxidant concepts.

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Therefore, they can be linear or branched, and have varying degrees of polymerization (DP) ranging from three to approximately 200 fructosyl units (Hendry, 1993; van Arkel et al., 2013). Fructan constitute the main carbon (C) reserve in a number of flowering plant species, which are stored in different organs and periods depending on the plant group. In dicots they are usually found as long-term C stores that accumulate in taproots and tubers, in contrast to monocot plants where fructans act as short-term C reserves in aboveground organs (van Arkel et al., 2013; Van den Ende, 2013).

Their utilization as storage CHOs in certain plants is advantageous considering that, contrary to starch, fructan biosynthesis is not interrupted by chilling temperatures. Fructans also offer the possibility of storing larger amount of photo-assimilates per unit issue volume without creating a damaging osmotic effect. Moreover, fructans storage in the vacuoles reduces physical strain in photosynthetic cells and favors C fixation and fructan subsequent utilization, initiated via a single enzymatic process involving fructan exohydrolases (FEHs). Agile fructan release impacts key plant processes such as C remobilization or osmotic regulation needed for flower aperture, grain filling or regrowth following defoliation and early spring growth (Van den Ende, 2013; Martínez-Noël et al., 2015; Gasperl et al., 2016). Fructan can also protect the cell under duress. The latter stems from the enhanced membrane stability and decreased membrane permeability provided during often-adverse conditions, such as freezing and cellular dehydration, by fructan's insertion between the head-groups of membrane lipids (Tarkowski and Van den Ende, 2015). An additional contribution emanates from the proposed role that fructans play in maintaining the reactive oxygen species (ROS) homeostasis of the cell during stress conditions. More importantly, fructans have the ability to scavenge the highly reactive and toxic hydroxyl radicals (•OH) that accumulate around vacuolar or chloroplast membranes (Tarkowski and Van den Ende, 2015; Passardi et al., 2004; Doltchinkova et al., 2013; Keunen et al., 2013). Not surprisingly, fructans are mostly found in plants that thrive in temperate and arid climates prone to periodical drought or freezing episodes (Hendry, 1993). Likewise, their natural or manipulated accumulation in various plant species, including many that don't naturally accumulate fructans, has been associated with tolerance to drought, salt, and freezing stress conditions (van Arkel et al., 2013; Van den Ende, 2013; Valluru, 2015).

Closely linked to their role in development and stress responses is the emerging experimental evidence showing that fructan metabolism in plants is embedded in the active crosstalk existing between sugar signaling and hormonal networks. A recent model proposes that both fructan biosynthesis and degradation processes involve the participation of abscisic acid (ABA), auxins (AUX), and ethylene (ET) in sucrose-specific pathways regulated by MYB transcription factors and a calcium-signaling network. This signaling network targets key enzymes in fructan biosynthesis or degradation, such as diverse fructosyltransferases (FTs), invertases and FEHs (Valluru, 2015). In this respect, we recently showed that exogenous ABA promoted fructan accumulation in *Agave tequilana* by inducing the expression of genes coding for two fundamental fructans biosynthetic enzymes such as sucrose: sucrose 1-fructosyltransferase (1-SST) and fructan: fructan 1-fructosyltransferase (1-FFT) (Suárez-González et al., 2014). The former predominantly catalyzes the synthesis of 1-kestose, from two molecules of sucrose, whereas 1-FFT catalyzes the transfer of fructosyl units from 1-kestose onto 1-kestose or higher DP fructan molecules (van Arkel et al., 2013). In addition, fructan accumulation and fructan biosynthetic gene expression in *A. tequilana* and wild *A. inaequidens*, were found to differentially respond to various other stimuli (e.g., sucrose and polyethylene glycol) and phytohormones, including kinetin (KIN), salicylic acid (SA), and methyl jasmonate (MeJA), the latter two which also mediate biotic stress responses. A more recent study reported, however, that pulse treatments with ABA, AUX, ET, gibberellic acid, or KIN on perennial ryegrass (*Lolium perenne* L.) had limited effects on the gene expression/activity of enzymes involved in fructans synthesis or degradation and the status of water-soluble carbohydrates (Gasperl et al., 2016). In this work, we investigated the shorter-term effect of exogenous sucrose (ExSuc), SA and MeJA, in addition to methyl viologen (MV), a ROS-generating herbicide, on fructan accumulation and composition. The expression levels of genes coding for enzymes involved in fructan biosynthesis or degradation or in C mobilization in plants, such as cell wall and vacuolar invertases was also examined. Biotic stress response marker genes whose expression in regulated predominantly by SA, were determined, as well. Our results support the proposed relation existing between fructan accumulation and protection against biotic and oxidative stress in plants. They also indicate that fructan accumulation and DP, and related gene expression, is dependent on the inducing stimulus applied in a time- and tissue-dependent manner.

2. Materials and methods

2.1. In vitro culture of Agave tequilana

In vitro cultivation of *Agave tequilana* (Weber) var. "Azul" plantlets was performed as described previously (Suárez-González et al., 2014). Briefly, agave plantlets were grown in liquid MS (Murashige and Skoog, 1962) medium in a conditioned plant growth chamber at a constant temperature of 25 °C ± 1 °C, under a 9 h light/15 h darkness photoperiod. The light source consisted of cold florescent lamps emitting a photosynthetic photon flow density of ~270 μmol m^{-2} s^{-1}.

2.2. Plant treatments

After 32 weeks, the agave plantlets were treated with different compounds, which were added directly to the MS media. The treatments included 8% exogenous sucrose (ExSuc), 1 mM salicylic acid (SA) (Suárez-González et al., 2014; Godoy-Hernández and Loyola-Vargas, 1997), 50 μM methyl jasmonate (MeJA) (Suárez-González et al., 2014; Silvakumar and Paek, 2005), and 5 μM methyl viologen (MV). This compound is a ROS generating herbicide usually employed to induce oxidative stress by accelerating the photo-production of O^{-}_{2} in photosystem I and by inhibiting the photo-reduction of monodehydroascorbate to ascorbate (Mano et al., 2001; Dalal et al., 2014). All chemicals were purchased from Sigma-Aldrich Chemical.

Leaves and stems of agave plantlets were sampled at 2, 4 and 6 days after treatments (DAT) were commenced. The sampling procedure involved the separation of all leaves from the plantlet body in order to reveal the stem. Then, the middle section of both stem and leaves (approximately 300 mg fresh weight [FW]) were thinned out by cutting their upper and lower edges. All plant tissues thus sampled were subsequently flash frozen in liquid N_2. Stem and leaf tissue from the three plantlets analyzed per treatment per time point were pooled together into single samples for subsequent analysis.

2.3. Fructan extraction and analysis

Fructans were extracted with water directly from the frozen leaf and stem samples. Aliquots of the fructan extracts were subsequently separated by thin-layer chromatography (TLC). These procedures were performed as described previously (Suárez-González et al., 2014). Standards of glucose (Glu), fructose (Fru) and sucrose were from Sigma-Aldrich, and the inulin series fructan (GFn,) were obtained from Megazime NTD. The neoseries fructans (FGFn) were identified according to the color of the bands and in accordance with the fructans present in agave, as reported by others (Praznik et al., 2013). Maltooligosaccharide standards (Mn) were from Sigma-Aldrich. Carbohydrate separation of interesting samples was also performed by high performance liquid chromatography (HPLC) using a Varian Prostar HPLC system (Agilent Technologies) coupled to an evaporative light scattering detector (ELSD), and fitted with an Imtakt UK amino column