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Expression of the 1-SST and 1-FFT genes and consequent fructan accumulation in *Agave tequilana* and *A. inaequidens* is differentially induced by diverse (a)biotic-stress related elicitors

Edgar Martín Suárez-González[a], Mercedes G. López[b], John P. Délano-Frier[b], Juan Florencio Gómez-Leyva[a,*]

[a] *Laboratorio de Biología Molecular, Instituto Tecnológico de Tlajomulco, Jalisco (ITTJ), km 10 Carretera a San Miguel Cuyutlán, CP 45640 Tlajomulco de Zúñiga, Jalisco, Mexico*
[b] *Unidad de Biotecnología e Ingeniería Genética de Plantas, Cinvestav-Unidad Irapuato, km 9.6, Libramiento Norte Carretera Irapuato-León, CP 36821 Irapuato, Guanajuato, Mexico*

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ABSTRACT

The expression of genes coding for sucrose:sucrose 1-fructosyltransferase (1-SST; EC 2.4.1.99) and fructan:fructan 1-fructosyltransferase (1-FFT; EC 2.4.1.100), both fructan biosynthesizing enzymes, characterization by TLC and HPAEC-PAD, as well as the quantification of the fructo-oligosaccharides (FOS) accumulating in response to the exogenous application of sucrose, kinetin (cytokinin) or other plant hormones associated with (a)biotic stress responses were determined in two *Agave* species grown *in vitro*, domesticated *Agave tequilana* var. azul and wild *A. inaequidens*. It was found that elicitors such as salicylic acid (SA), and jasmonic acid methyl ester (MeJA) had the strongest effect on fructo-oligosaccharide (FOS) accumulation. The exogenous application of 1 mM SA induced a 36-fold accumulation of FOS of various degrees of polymerization (DP) in stems of *A. tequilana*. Other treatments, such as 50 mM abscisic acid (ABA), 8% Sucrose (Suc), and 1.0 mg L^{-1} kinetin (KIN) also led to a significant accumulation of low and high DP FOS in this species. Conversely, treatment with 200 μM MeJA, which was toxic to *A. tequilana*, induced an 85-fold accumulation of FOS in the stems of *A. inaequidens*. Significant FOS accumulation in this species also occurred in response to treatments with 1 mM SA, 8% Suc, and 10% polyethylene glycol (PEG). Maximum yields of 13.6 and 8.9 mg FOS per g FW were obtained in stems of *A. tequilana* and *A. inaequidens*, respectively. FOS accumulation in the above treatments was tightly associated with increased expression levels of either the *1-FFT* or the *1-SST* gene in tissues of both *Agave* species.

Introduction

Agaves are perennial monocarpic plants that perform the Crassulacean Acid Metabolism (CAM), which is characteristic of most xerophytes (Pimienta-Barrios et al., 2005). In CAM plants, stomatal opening and CO_2 uptake occur primarily at night when lower temperatures greatly reduce water loss (Nobel, 1990). Agaves are native of the American continent. Mexico, in particular, has the highest biodiversity of Agave plants, with approximately 75% of the 210 known species being indigenous to this country. Agaves have been traditionally used for the production of food, beverages and fibers (García-Mendoza, 2007). Two outstanding *Agave* species employed for industrial use in the Mexican state of Jalisco are *Agave tequilana* and *A. inaequidens*. The first is used for the production of tequila, a distilled beverage that is known and appreciated world-wide, whereas *A. inaequidens*, which grows wild in the Western mountainous range of Jalisco, is employed in the manufacture of handcrafted ropes, known as *sogas finas* or *charro*

Abbreviations: 1-FFT, fructan:fructan 1-fructosyltransferase; 1-SST, sucrose:sucrose 1-fructosyltransferase; 6G-FFT, fructan:fructan 6-G-fructosyltransferase; 6-SFT, sucrose:fructan 6-fructosyltransferase; ABA, abscisic acid; BLAST, basic local alignment search tool; CAM, Crassulacean acid metabolism; CS, cold-stress; CTRL, control; DAT, days after treatment; DP, degree of polymerization; FEH, fructan exohydrolase; FOS, fructo-oligosaccharides; Fru, fructose; Glc, glucose; Hi, high; HPAEC-PAD, high-performance anion exchange chromatography coupled with pulsed amperometric detection; Inu, inulin; KIN, kinetin; L, leaf; Lo, low; Mal, maltose; MeJA, methyl jasmonate; MS, Murashige and Skoog medium; PEG, polyethylene glycol; S, stem; SA, salicylic acid; SLN, salicin; SPS, sucrose phosphate synthase; sqRT-PCR, semi-quantitative reverse transcriptase-polymerase chain reaction; Suc, sucrose; TLC, thin layer chromatography; WSC, water soluble carbohydrates.

* Corresponding author. Tel.: +52 3337724426; fax: +52 3337724426.
E-mail addresses: jfleyva@hotmail.com, jfgomez@ittlajomulco.edu.mx (J.F. Gómez-Leyva).

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lariats, and also for the production of a spirit known as *Raicilla*, whose consumption has been gradually increasing in the past few years (Valenzuela-Zapata et al., 2011). A salient characteristic in *Agave* plants is their content of water-soluble carbohydrates (WSC), which can represent up to 80% of the total dry weight (Nobel, 1990). These carbohydrates are mainly fructans, inulin (Inu) and fructo-oligosaccharides (FOS), which represent more than 60% of total WSC, while the rest is mostly consisting of glucose (Glc), fructose (Fru) and sucrose (Suc). The WSC can be used commercially for the production of honey and flour of agave (Mancilla-Margalli and López, 2006).

Fructans are fructose polysaccharides derived from sucrose. They vary in length, from the trisaccharides 1-kestotriose, 6-kestotriose or neokestotriose to polymers having hundreds of fructose units. Fructans have been classified into six groups depending on the linkage type between the fructosyl residues and the position of the glucose (Vijn and Smeekens, 1999; Mancilla-Margalli and López, 2006): (1) inulins, which are characteristic of members of the Asteraceae such as chicory and the Jerusalem artichoke, are linear fructan polymers having a terminal glucose residue and β-(2-1) glycosidic bonds; (2) levans, in which the fructosyl units are connected through β-(2-6) linkages, are abundant in grasses such as *Phleum pretense* and *Lolium perenne*; (3) graminans, which have both β-(2-1) and β-(2-6) linkages and are found in wheat, barley and some members of the Asparagales; (4) inulin neoseries, which are characteristic of fructans in onion and asparagus, have an internal glucose bonded at positions C1 and C6 to two linear fructosyl chains linked by β-(2-1) bonds; (5) levan neoseries, found in oats and a few species belonging to the Poales, are polymers formed by β-(2-1)- and β-(2-6)-linked fructofuranosyl units on either end of a central sucrose molecule, and (6) agavins, categorized as ramified graminans and neo-fructans, have β-(2-1) and β-(2-6) bonds and have been found in the *Agave* and *Dasylirion* genera (Mancilla-Margalli and López, 2006). Fructans are water soluble reserve carbohydrates found in several angiosperms adapted to regions having seasonal cold and dry periods (Vijn and Smeekens, 1999). Due to their water solubility and resistance to crystallization at freezing temperatures, they undergo biosynthesis at low temperatures, stabilize phospholipid membranes, adjust the osmotic pressure by hexose sugars release (Valluru et al., 2008; Kusch et al., 2009) and act as scavengers of reactive oxygen species (ROS) in the vicinity of vacuolar membranes (Peshev et al., 2013). They are believed to contribute to the protection against abiotic stress caused by cold (Pollock et al., 1988; Hisano et al., 2008), excessive salinity (Kerepesi and Galiba, 2000), drought (De Roover et al., 2000; Livingston et al., 2009) and/or oxidative stress (Peshev et al., 2013). Increased stress tolerance has been achieved by triggering fructan accumulation *via* transgenic approaches in rice (Kawakami et al., 2008), tobacco (Pilon-Smits et al., 1995; Li et al., 2007), sugar beet (Pilon-Smits et al., 1999) and potato (Knipp and Honermeier, 2006). Fructans are viewed as prebiotics, since they show health benefits to animals and humans, including improved absorption of inorganic nutrients, especially calcium, reduced incidence of colon cancer and lower blood concentrations of glucose, triglycerides and fatty acids (Urías-Silvas et al., 2008; Gomez et al., 2010).

Fructans are synthesized and stored in the vacuoles of plant cells. Synthesis involves the action of different fructosyltransferases such as sucrose:sucrose 1-fructosyltransferase (1-SST; EC 2.4.1.99), sucrose:fructan 6-fructosyltransferase (6-SFT), fructan:fructan 1-fructosyltransferase (1-FFT; EC 2.4.1.100) and fructan:fructan 6G-fructosyltransferase (6G-FFT) (Frehner et al., 1984; Darwen and John, 1989; Wiemken and Smeekens, 1997; Vijn and Smeekens, 1999). Fructan breakdown is catalyzed by several fructan exohydrolases, including fructan 1-exohydrolases (1-FEHs; EC 3.2.1.153), 6-FEH and 6&1-FEH that degrade inulins (Van den Ende et al., 2000, 2001, 2003a), levans (Van den Ende et al., 2003b; Van Riet et al., 2006) and graminans (De Coninck et al., 2005), respectively.

Apart from a report describing how the addition of phytohormones improved growth and fructan accumulation in the medicinal herb *Symphytum officinale* cultured *in vitro* (Haaß et al., 1991), not much information regarding the effect of tissue culture conditions on the levels of fructans in fructan-accumulating plants is available. In this work, we tested whether the addition of different stress-related compounds, including the osmotic/drought stress-associated compounds polyethylene glycol (PEG) (Turkan et al., 2005; Landjeva et al., 2008) and abscisic acid (ABA) as well as the more universal elicitors salicylic acid (SA) and jasmonic acid methyl ester (MeJA), could induce the accumulation of fructans in two *Agave* species, *A. tequilana* and *A. inaequidens*, grown *in vitro*. Jasmonates and SA, apart from their association with biotic stress, such as pathogen infection, wounding and/or insect herbivory, often play a central role in the regulation of the biosynthesis of secondary metabolites, including terpenoids, alkaloids, phenylpropanoids, and antioxidants. SA has also been associated with various physiological processes in higher plants, including growth, thermogenesis, flower induction, nutrient uptake, ethylene biosynthesis and stomatal movement (Hayat and Ahmad, 2007), and it is an important signaling component in abiotic stress responses regarding tolerance to drought, salt, freezing, ozone and UV-B (Janda et al., 1999; Singh and Usha, 2003).

We report that both SA and MeJA were able to greatly increase both low- and high-degree of polymerization (DP) FOS in *Agave*. The effect was species-specific, since MeJA concentrations that were toxic to *A. tequilana* induced the highest accumulation of low and high DP FOS in *A. inaequidens*. Moreover, increased FOS accumulation of varying DP was found to be tightly correlated with the induced expression of the *1-SST* and *1-FFT* genes. To the best of our knowledge, this is the first report describing the induced accumulation of FOS and concomitant expression of fructan biosynthetic genes by SA, MeJA and other elicitors in *Agave*.

Materials and methods

In vitro culture of Agave sp.

The regeneration of *in vitro* plants of *Agave tequilana* Weber var. azul was realized by indirect organogenesis according to the protocol by Valenzuela-Sánchez et al. (2006) using stem tissue from ramets emerging from mother *A. tequilana* plants. Individual regenerated shoots were carefully separated and placed on solid MS (Murashige and Skoog, 1962) medium without growth regulators to induce root formation. The plantlets were then transferred to liquid MS medium, which was changed every month until they reached a size of 15 cm. The whole process was completed in 12 months (see Appendix S1A in Supporting Information). The jars were incubated in a plant growth chamber (Lab-Line, Biotronette, Mark IV, Barnstead International) operating at 25 °C ± 1 °C under a 9 h light/15 h dark photoperiod with cold fluorescent white lights emitting a photon flux density of ~270 μmol m^{-2} s^{-1}.

Seeds from *A. inaequidens* Koch subsp. inaequidens were obtained from plants grown in the wild in the "Cerro Viejo" mountains (20°23′40″ N 103°25′41″ W) located in the municipality of Tlajomulco, Jalisco, Mexico. They were superficially sterilized with 96% ethanol for 1 min, transferred to 1.2% solution of sodium hypochlorite for 1 h and rinsed with sterile distilled water. Ten to twenty seeds were placed in sterile glass flasks containing MS medium and kept in a plant growth chamber operating as described above. After germination (3–7 d), the seedlings were allowed to grow to a size of approximately 5 cm and then transferred to liquid

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Table 1
Primers used for RT-PCR amplification of fructosyl transferase and 18S rRNA genes in *Agave* spp.

Code	Primer sequence	Tm (°C)	Fragment (bp)	Accession number
1-SST-At F	5′ CCATGGTTCCTGACCATTGGTACG 3′	50	700	DQ535031
1-SST-At R	5′ TCGGTCTCNCCAACRTANSCCCA 3′			
1-FFT-At F[a]	5′ TGGCACAGGTCGTCAATCTT 3′	50	600	EU026119
1-FFT-At R[a]	5′ TTCCCCAACATACCCCCATAGCAC 3′			
1-SST-Ai F	5′ GGGSCCACGCCGTITCYAAGGA 3′	50	600	DQ535031
1-SST-Ai R	5′ GTCSMTGCTCKCCTTRAGCACG 3′			
18S-At-F[a]	5′ GCTACCACATCCAAGGAAG 3′	60	135	GQ983553
18S-At-R[a]	5′ TCGTTAAGGGATTTAGATTGT 3′			

At, *A. tequilana*; Ai, *A. inaequidens*; I, deoxyInosine; K, T+G; M, A+C; R, A+G; S, C+G; Y, C+T.
[a] Used in *A. inaequidens* RT-PCR amplification.

MS medium until they reached 15 cm. The whole process was completed in 7 months (see Appendix S1B in Supporting Information).

Plant treatments

Plantlets established in liquid MS media (control, CTRL) were used in all experiments. All experiments were performed in the growth chamber operating under the conditions described above and had a duration of 24 and 42 d, except for the cold-stress (CS) treatment, in which the plantlets were first exposed to freezing temperature (0 °C) for 10 d and subsequently allowed to acclimatize at 25 °C for the duration of the experiment (Kawakami et al., 2008). Different substances were added to the MS media: 8% sucrose (SUC), 5 and 10% polyethyleneglycol 10,000 (PEG-Lo [low] and PEG-Hi [high], respectively) (Pilon-Smits et al., 1995) and 10 and 50 μM abscisic acid (ABA-Lo and ABA-Hi) (Onofre et al., 2003). They were added to induce osmotic stress and to simulate drought conditions. Salicylic acid (SA-Lo and SA-Hi) and salicin (SLN-Lo and SLN-Hi), a salicyl alcohol glucoside often used as a SA precursor, were applied at 1 and 20 mM (Godoy-Hernández and Loyola-Vargas, 1997), whereas methyl jasmonate (MeJA-Lo and MeJA-Hi) was applied at 50 and 200 μM (Silvakumar and Paek, 2005). These 3 additives were used to test the effect of compounds usually associated with biotic stress responses. Finally, the effect of kinetin, a plant growth regulator, was tested at 0.5 and 1.0 mg L^{-1} (KIN-Lo and KIN-Hi) (Haaß et al., 1991).

Fructo-oligosaccharide extraction

The agave plantlets were sampled 24 and 42 d after treatment (DAT). Samples of 0.3 g were taken from fresh leaf (L) and stem (S) tissues, flash frozen in liquid N_2 and ground to a fine powder with mortar and pestle. The powdered tissues were transferred to 1.5 mL plastic microtubes and one volume of distilled sterile water was added. The mixture was briefly vortexed (Thermolyne, Maxi Mix II, Barnstead International) and maintained in a water bath at 90 °C for 5 min (Pilon-Smits et al., 1995). Thermal incubation was followed by centrifugation at 14,000 rpm for 10 min in a table microfuge (LABNET, Spectrafuge 16 M, LabNet International Inc.). The supernatants were recovered and stored at −20 °C until required.

Fructo-oligosaccharide characterization by thin layer chromatography

Aliquots of the aqueous extracts were analyzed to determine the type and size of fructans present. One μL aliquots were applied on Silicagel F1500 plates (Sigma–Aldrich Chemical Co.) in addition to two standard mixtures at 10 mg mL^{-1} composed of Glc and maltose (Mal), as well as Fru, Suc, 1-kestotriose (DP3), 1,1-kestotetraose (DP4), 1,1,1-kestopentaose (DP5) and inulin from chicory (*Cichorium intybus*). The plates were run for 45 min with a mobile phase composed of ethyl acetate: acetic acid: methanol: water (12:3:3:2, v/v/v/v). After drying at room temperature for 10 min, the plates were developed by spraying with 4% diphenylamine, 4% aniline dissolved in acetone and 10% phosphoric acid as described by Anderson et al. (2000), followed by heating at 100 °C for 10 min. To allow full development, the plates were sprayed a second time and heated for 5 min.

Fructo-oligosaccharide identification and quantification by high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD)

This technique was employed to determine the presence and concentration of Glc, Fru, Suc, and FOS of varying DP. A 1:250 dilution of the aqueous fructan extracts in MilliQ-grade water (Sigma) was made. The diluted solutions were filtered through a 0.22 μm membrane (Millipore, Billerica, MA) directly into 5-mL lidded flasks, were briefly vortexed and subsequently degassed by sonication (Lab-Line, Ultrasonic LC60H) for 15 min. The samples were subsequently injected into a high-performance anion exchange chromatography apparatus coupled with pulsed amperometric detection (HPAEC-PAD) (Dionex ICS-3000; Thermo Fisher Scientific Inc.) and equipped with a guard column attached to a CarboPac PA100 column (Dionex, 4 mm × 250 mm). The running conditions were as follows: column temperature 25 °C; injected volume 25 μL eluted at a flow rate of 0.8 mL min^{-1}. Samples were separated using a 75-min linear gradient from 0 to 100% of eluent A. The eluents used were: (A) 150 mM NaOH and (B) 150 mM NaOH plus 500 mM sodium acetate. The potentials applied for detection by amperometric pulse E1 (400 ms), E2 (20 ms), E3 (20 ms) and E4 (60 ms) were +0.1, −2.0, +0.6 and −0.1 V, respectively. Glc, Fru, Suc, 1-kestotriose, 1,1-kestotetraose and 1,1,1-kestopentaose molecules were identified by comparison with commercial standards.

Total fructo-oligosaccharide determination

An enzymatic assay kit for the measurement of total fructo-oligosaccharides (AOAC Method 999.03) was employed following the manufacturer's instructions (Megazyme).

Cloning of the partial cDNAs of the 1-SST and 1-FFT genes

Total RNA was extracted using the Trizol reagent (Invitrogen) starting from 10 mg of frozen foliar or stem tissue taken from *in vitro* cultured plants of *A. tequilana* and *A. inaequidens*. The manufacturer's instructions were followed except for an additional centrifugation step for the elimination of polysaccharides, proteins and residual extracellular debris. cDNA synthesis was performed with 2 μg of total RNA employing random hexamers as described in the Super Script™ III First-Strand Synthesis for RT-PCR kit (Invitrogen). The partial cDNA sequences corresponding to the *1-SST* and *1-FFT* genes were obtained using the specific primers listed in Table 1. The amplification conditions employed were the following: initial denaturalization at 94 °C for 2 min, followed by 30 cycles

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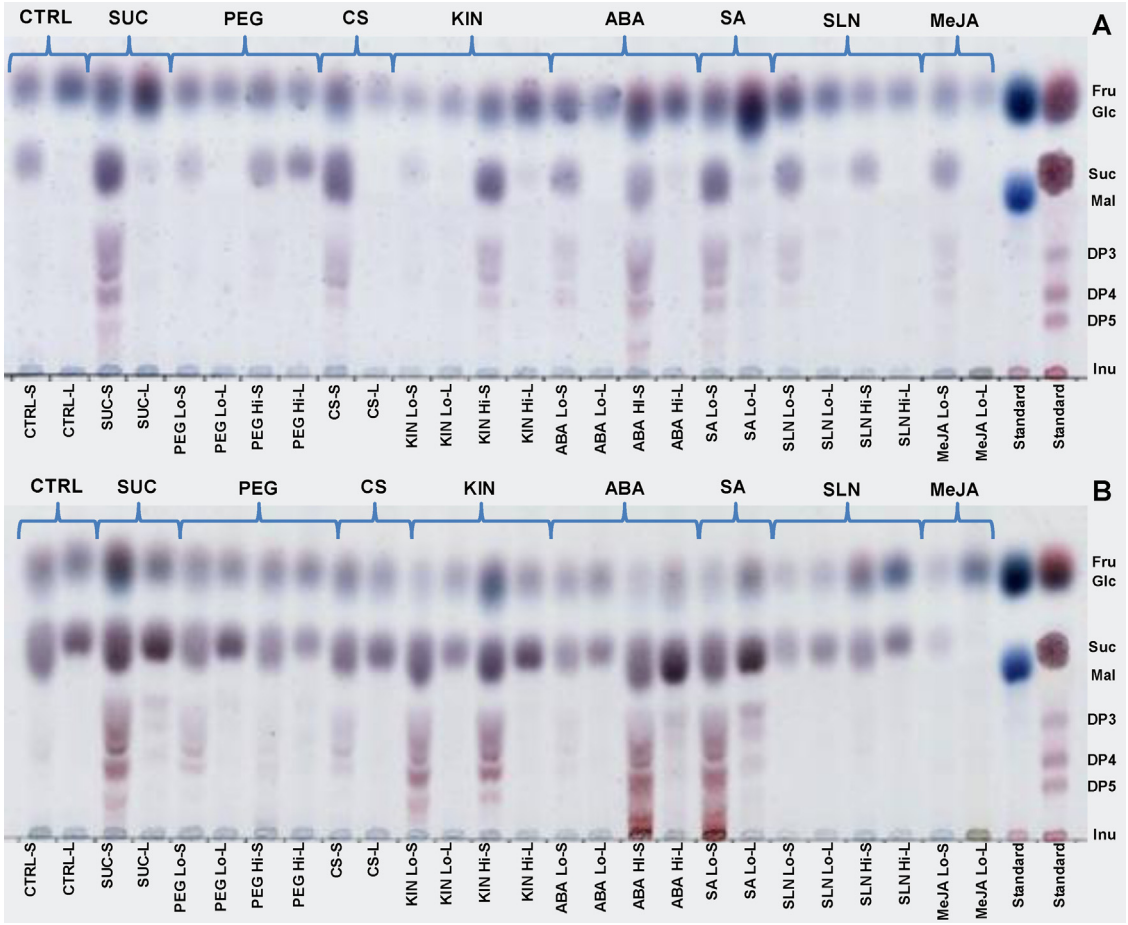

Fig. 1. Thin layer chromatography analysis of fructans extracted from stem (S) and leaf (L) tissue of *in vitro*-cultured *A. tequilana* plantlets at (A) 24 and (B) 42 d after treatment (DAT). Treatments included the application of cold stress (CS: 10 d at 0 °C + 14 or 32 d at 25 °C) or the addition to the medium of 8% sucrose (SUC), polyethylene glycol 10,000 (5% [PEG-Lo] or 10% [PEG-Hi]), kinetin (0.5 mg l^{-1} [KIN-Lo] or 1 mg l^{-1} [KIN-Hi]), abscisic acid (10 μM [ABA-Lo] or 50 μM [ABA-Hi]), salicylic acid (1 mM [SA-Lo]), salicin (1 mM [SLN-Lo] or 20 mM [SLN-Hi]) or methyl jasmonate (50 μM [MeJA-Lo]). Untreated controls are represented as CTRL. The markers represent the mobility of fructose (Fru), glucose (Glc), sucrose (Suc), maltose (Mal), 1-kestose (DP3), 1,1-kestotetraose (DP4), 1,1,1-kestopentose (DP5) and inulin (Inu) from chicory.

consisting of a denaturalization step at 94 °C for 1 min, a hybridization step at 60 °C for 1 min, and an extension step at 72 °C for 1 min. The run was completed with a final extension step at 72 °C for 7 min. The cDNA fragments synthesized were purified using the Wizard® SV Gel and PCR Clean-up System (Promega) and subsequently linked into the PCR®4-TOPO vector (Invitrogen). The resulting construct was employed to transform chemically competent *Escherichia coli* Top 10 cells according to the instructions described in the TOPO TA Cloning® Kit for Sequencing (Invitrogen). The resulting sequences were verified against Gen Bank databases using BLAST (Basic Local Alignment Search Tool) programs (Altschul et al., 1990).

Gene expression assays

The level of expression of the *1-SST* and *1-FFT* genes in stems and leaves of *A. tequilana* and *A. inaequidens* plantlets cultured *in vitro* and subjected to different treatments and elicitors was performed by semi-quantitative reverse transcriptase-polymerase chain reaction (sqRT-PCR) assays. Total RNA isolation, cDNA synthesis and PCR amplification for each gene was performed as described earlier. The amplified bands were separated in 1.2% agarose gels with ethidium bromide and visualized under UV light at 302 nm. Controls without reverse transcriptase were carried out in order to exclude contamination by genomic DNA. The images were analyzed in a DOC-PRINT DP-001-FDC gel photo-documentation system (Vilber Lournat). The semi-quantitative gene expression assays were analyzed and quantified based on the staining intensities of the corresponding bands as assessed using the ImageJ program (http://rsb.info.nih.gov/ij/index.html, version 1.43). The 18S rRNA gene product was used as loading control.

Experimental design and statistical analysis

A completely randomized experimental design was employed for the two agave species, with 15 treatments and two replicates in two different events. An experimental unit consisted of a flask containing two plantlets. The total fructo-oligosaccharide results were square-root-transformed ($\sqrt{[x+0.5]}$) (Montgomery, 2004). Data were analyzed by one-way ANOVA, and a multiple comparison procedure with the Tukey's test was performed to find significant differences between means. All tests were conducted using the STATGRAPHICS Centurion XV software package (Statpoint Inc, 2007). Differences at $p < 0.01$ were considered as statistically significant.

Results

Fructo-oligosaccharide analysis of in vitro cultured agave plantlets subjected to cold stress or treated with phytohormones or (a)biotic stress elicitors

Thin-layer-chromatography analyses were performed to determine the pattern of FOS accumulation in agave plantlets subjected to various treatments. The results shown in Fig. 1A show that no

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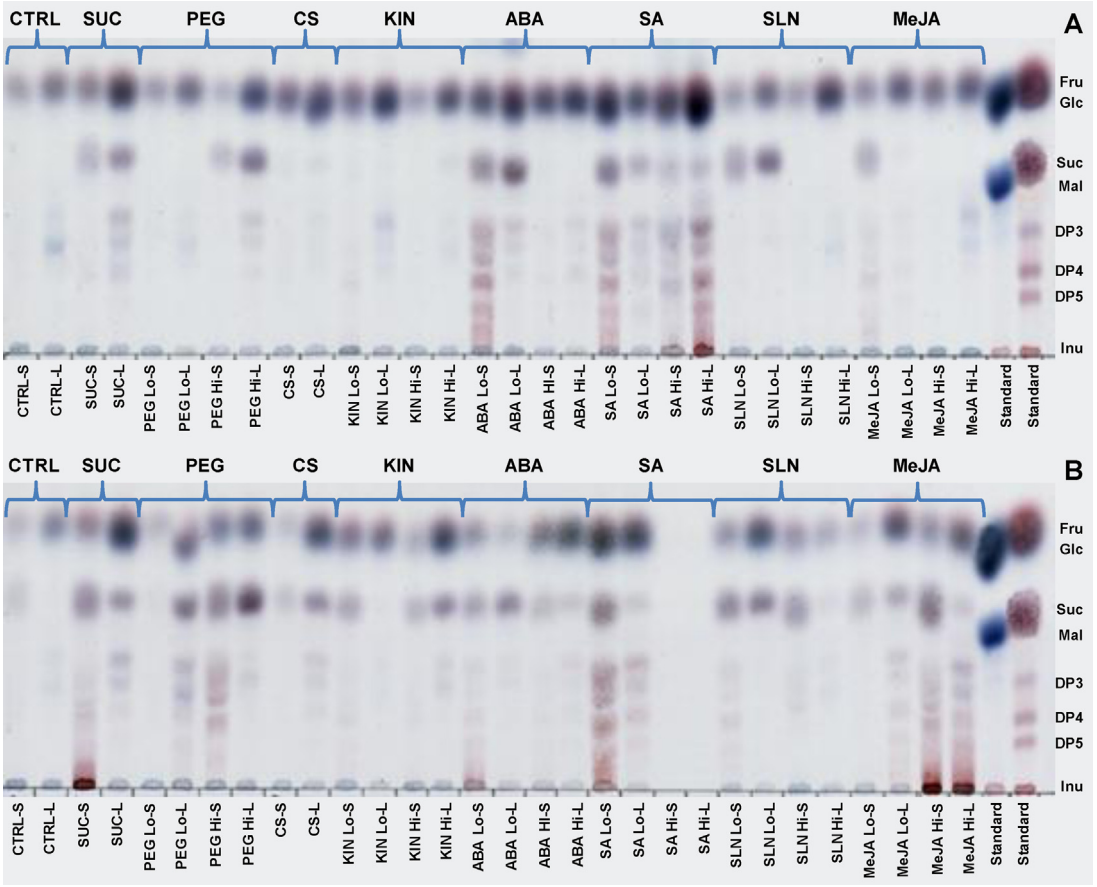

Fig. 2. Thin layer chromatography analysis of fructans extracted from stem (S) and leaf (L) tissue of *in vitro*-cultivated *A. inaequidens* plantlets at (A) 24 and (B) 42 DAT (no sugars were loaded for SA-Hi treated plants since this treatment was lethal). Treatments included the application of cold stress (CS: 10 d at 0 °C + 14 or 32 d at 25 °C) or the addition to the medium of 8% sucrose (SUC), polyethylene glycol 10,000 (5% [PEG-Lo] or 10% [PEG-Hi]), kinetin (0.5 mg l^{-1} [KIN-Lo] or 1 mg l^{-1} [KIN-Hi]), abscisic acid (10 μM [ABA-Lo] or 50 μM [ABA-Hi]), salicylic acid (1 mM [SA-Lo] or 20 mM [SA-Hi]), salicin (1 mM [SLN-Lo] or 20 mM [SLN-Hi]) or methyl jasmonate (50 μM [MeJA-Lo] or 200 μM [MeJA-Hi]). Untreated controls are represented as CTRL. The markers represent the mobility of fructose (Fru), glucose (Glc), sucrose (Suc), maltose (Mal), 1-kestose (DP3), 1,1-kestotetraose (DP4), 1,1,1-kestopentose (DP5) and inulin (Inu) from chicory.

FOS accumulated in control *A. tequilana* plantlets sampled 24 d after treatment (DAT) irrespective of the tissues sampled. Conversely, FOS with a DP of 3–5 accumulated 24 DAT in stems receiving the following treatments: SUC, KIN-Hi, cold stress (CS), ABA-Lo and ABA-Hi, SA-Lo, SLN-Lo and MeJA-Lo. Treatments with the higher concentrations of SA and MeJA were toxic to the *A. tequilana* plantlets. In leaves, a weak FOS accumulation was only observed in *A. tequilana* plantlets treated with SA-Lo. At 42 DAT, stems of control plants showed a weak accumulation of low DP FOS. The accumulation of low DP FOS became evident (*i.e.* PEG-Lo, CS) or more intense (SUC, KIN-Lo, KIN-Hi) in stems of *A. tequilana* (Fig. 1B). Low DP FOS were also detected in leaves of SUC-treated plantlets. The ABA-Hi and SA-Lo treatments led to a higher accumulation of low DP FOS, whereas FOS having a higher DP were also detected (Fig. 1B). Curiously, low DP FOS decreased in stems of CS treated plantlets sampled at this late time point, indicating that FOS levels were gradually reduced in the absence of the stress condition. A more prominent reduction in FOS was observed in stems of SLN-Lo- and MeJA-Lo-treated plants (Fig. 1B).

Several differences in the pattern of FOS accumulation were observed in *A. inaequidens* with respect to *A. tequilana*. First, a weak low DP FOS accumulation was detected in leaves of control plantlets, but not in stems at 24 DAT, while at 42 DAT no FOS accumulation was observed in any tissue. A similar low accumulation of low DP FOS in leaves, but not in stems, was observed 24 DAT in *A. inaequidens* plantlets treated with SUC and both levels of PEG and KIN (Fig. 2A). Higher levels of low DP FOS were also detected at this time point in leaves of plantlets subjected to ABA-Lo, SA-Lo and SA-Hi treatments, although in two of the treatments (ABA-Lo and SA-Lo), levels in stems were higher. In the SA-Hi treatment, the transiently induced foliar accumulation of high DP FOS (Fig. 2A) was particularly strong. Second, *A. inaequidens* appeared to be less sensitive to stress caused by SA-Hi and MeJA-Hi than *A. tequilana*. The accumulation of high DP FOS was observed at 42 DAT in stems of SA-Lo-treated and SUC-treated plantlets and in both tissues of plantlets treated with MeJA-Hi (Fig. 2B). In contrast to SA, SLN did not have the same effect on FOS levels, since these treatments showed a low FOS accumulation with both levels of SLN in stem tissue at 42 DAT (Fig. 2B). On the other hand, a low accumulation of low DP FOS was observed at 24 and 42 DAT in stems and leaf tissues of ABA-Hi-treated plantlets (Fig. 2A and B).

The TLC results were corroborated by the enzymatic FOS assays and HPAEC-PAD separations. Thus total FOS accumulation in *A. tequilana* occurred predominantly in stems and increased with time (42 DAT > 24 DAT), with SUC, ABA-Hi and SA-Lo being the most efficient inducing treatments (Fig. 3A), whereas FOS accumulation in leaf tissues was slightly lower in CTRL plants and higher in ABA-Hi treatments (Fig. 3B). Slightly lower total FOS levels were detected in the stems of *A. inaequidens* (Fig. 4A). In the latter species, SUC and SA-Lo were again efficient elicitors of a time-dependent FOS accumulation, but were surpassed by MeJA-Hi, which induced the highest FOS levels in stems at 42 DAT. An earlier FOS accumulation in stems of this species was also induced by ABA-Lo and MeJA-Lo, being higher at 24 DAT and declining afterwards (Fig. 4A). In

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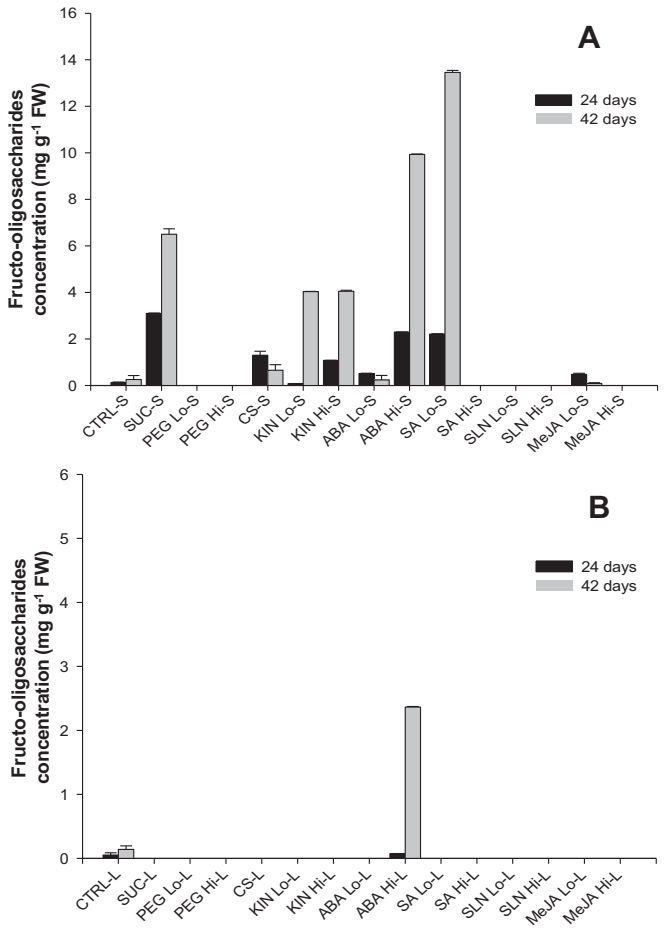

Fig. 3. Enzymatic assay for determination of total fructan concentration in stem (A) and leaf (B) tissue of *in vitro*-grown *A. tequilana* at 24 (black columns) and 42 (gray columns) DAT. Vertical bars show mean concentration ± SD of fructan in mg ml^{-1} FW. Treatments included the application of cold stress (CS: 10 d at 0 °C + 14 or 32 d at 25 °C) or the addition to the medium of 8% sucrose (SUC), polyethylene glycol 10,000 (5% [PEG-Lo] or 10% [PEG-Hi]), kinetin (0.5 mg l^{-1} [KIN-Lo] or 1 mg l^{-1} [KIN-Hi]), abscisic acid (10 μM [ABA-Lo] or 50 μM [ABA-Hi]), salicylic acid (1 mM [SA-Lo] or 20 mM [SA-Hi]), salicin (1 mM [SLN-Lo] or 20 mM [SLN-Hi]) or methyl jasmonate (50 μM [MeJA-Lo] or 200 μM [MeJA-Hi]). Untreated controls are represented as CTRL, (S) steam, (L) leaf.

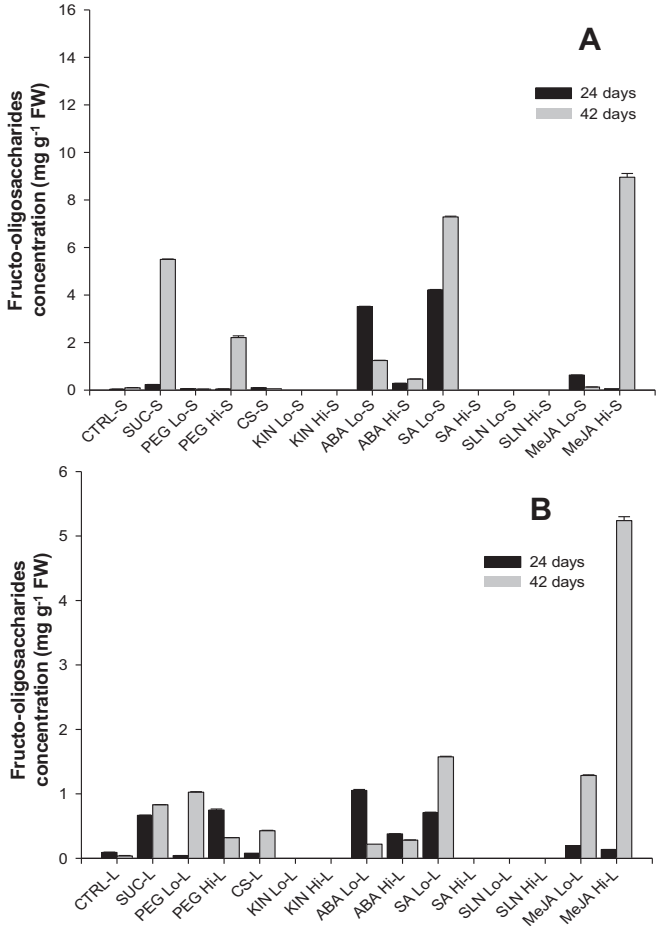

Fig. 4. Enzymatic assay for the determination of total fructan concentration in stem (A) and leaf (B) tissue of *in vitro*-grown *A. inaequidens* at 24 (black columns) and 42 (gray columns) DAT. Vertical bars show mean concentration ± SD of fructan in mg ml^{-1} FW. The treatments are the same as those described in Fig. 3. Untreated controls are represented as CTRL, (S) steam, (L) leaf.

contrast to *A. tequilana*, significant levels of FOS also accumulated in leaves of *A. inaequidens*, although at lower levels than in the stems (Fig. 4B). Similarly to stems, MeJA-Hi and SA-Lo were the most efficient elicitors of foliar FOS accumulation at 42 DAT.

The presence of FOS having a DP higher than 6, as determined by HPAEC-PAD, coincided with those treatments capable of inducing the highest total FOS levels. Thus, higher DP FOS were detected at 24 and 42 DAT predominantly in *A. tequilana* plantlets treated with SUC, ABA-Hi and SA-Lo (Fig. 5A and B). Conversely, increased levels of Glu, Fru and Suc were detected in leaf tissues at 24 DAT, but were reduced to undetectable levels at 42 DAT (see Appendix S4–6 in Supporting Information).

Higher DP FOS were detected in stems of *A. inaequidens* plantlets examined 42 DAT, particularly in those treated with 8% SUC, SA-Lo and MeJA-Hi (Fig. 6A and B). Additionally, the HPAEC-PAD analysis also showed that FOS abundance was higher at 24 DAT in plants treated with ABA-Lo and MeJA-Lo (see Appendix S8 in Supporting Information). This technique also reflected the obviously stronger FOS-inducing capacity that CS (compare Appendix S2 and S7 in Supporting Information) and KIN (compare Fig. 5B and Appendix S3 with S8 in Supporting Information) had on stems of *A. tequilana* when compared to those of *A. inaequidens*, which responded very weakly to these treatments. On the other hand, and unlike *A. tequilana*, relatively elevated levels of high DP FOS were detected at 42 DAT in leaves of *A. inaequidens* plantlets treated with MeJA-Hi (see Appendix S11 in Supporting Information).

The expression patterns of the 1-SST and 1-FFT genes coincided with those of FOS accumulation in agave

The partial cDNA sequences cloned using a PCR amplification strategy based on the use of specific primers are listed in Table 1. With respect to *A. tequilana*, the cDNA fragments corresponding to the *1-SST* and *1-FFT* genes showed 99% and 100% homologies with the *1-SST* (Accession No. DQ535031.1), and *1-FFT* (Accession No. EU026119.1) genes of *A. tequilana* reported in the GenBank databases. A similar result was obtained with the *1-SST* and *1-FFT* cDNA fragments isolated from *A. inaequidens*, which showed a 99% homology with its counterpart in *A. tequilana*. A semi-quantitative analysis of the expression pattern of these genes in response to the different treatments examined was performed once their correct identity was confirmed.

The overall results obtained in *A. tequilana* showed that, independently of the treatments, the expression level of the *1-FFT* gene had a tendency to be much higher in stems, whereas the expression level of the *1-SST* gene tended to be similar in leaves and stems, in accordance with the results reported by Cortés-Romero et al. (2012). This tendency was observed at both sampling dates

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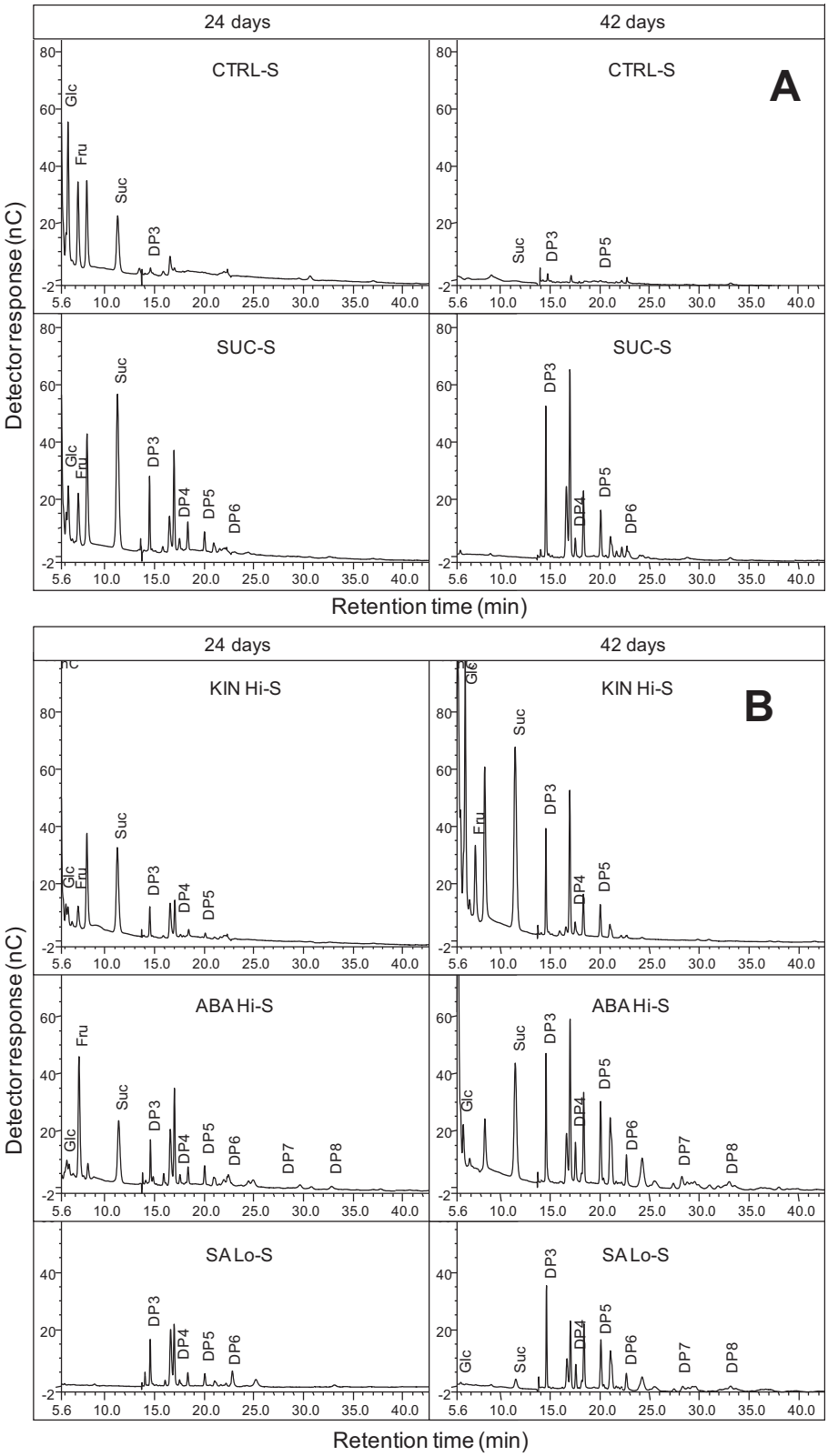

Fig. 5. High-performance anion exchange chromatography coupled with pulsed amperometric detection analysis of water soluble carbohydrates extracted from stem tissue of *A. tequilana* plantlets grown *in vitro* at 24 and 42 DAT, with emulators of abiotic (A) and biotic stress (B). Treatments included the addition to the medium of 8% sucrose (SUC), kinetin (1 mg l^{-1} [KIN-Hi]), abscisic acid (50 μM [ABA-Hi]) or salicylic acid (1 mM [SA-Lo]). Untreated controls are represented as CTRL. Compounds were detected using external standard: Glc, Fru, Suc, 1-kestotriose (DP3), 1,1-kestotetraose (DP4), 1,1,1-kestopentaose (DP5) and raftilose for DP higher than 5.

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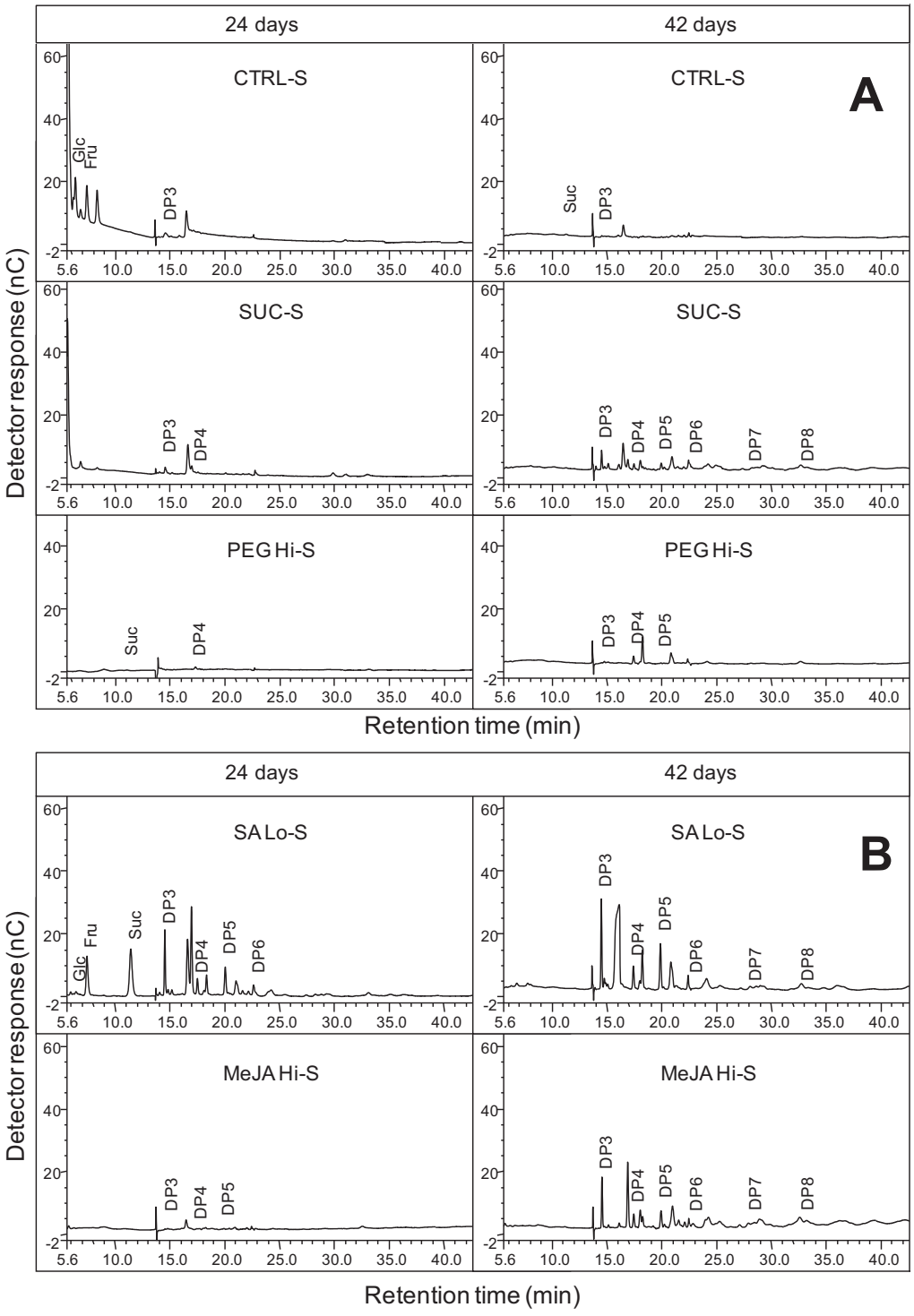

Fig. 6. High-performance anion exchange chromatography coupled with pulsed amperometric detection analysis of water soluble carbohydrates extracted from stem tissue of *A. inaequidens* plantlets grown *in vitro* at 24 and 42 DAT, with emulators of abiotic (A) and biotic stress (B). Treatments included he addition to the medium of 8% sucrose (SUC), polyethylene glycol 10,000 (10% [PEG-Hi]), salicylic acid (1 mM [SA-Lo]) or methyl jasmonate (200 μM [MeJA-Hi]). Untreated controls are represented as CTRL. Compounds were detected using external standard: Glc, Fru, Suc, 1-kestotriose (DP3), 1,1-kestotetraose (DP4), 1,1,1-kestopentaose (DP5) and raftilose for DP higher than 5.

examined (see below). Thus, higher expression levels of *1-FFT* detected at both 24 and 42 DAT in stems of *A. tequilana* plants treated with SUC, KIN-Hi, ABA-Hi and SA-Lo (Figs. 7A and 8A) coincided with an increased accumulation of both total FOS (Fig. 3A) and higher DP FOS (Figs. 1A, B and 5A, B). The same correlation was observed in the KIN-Lo treatment, but only after 42 DAT (compare Fig. 8A and C with Fig. 3A and Appendix S3 in Supporting Information), whereas it was not that evident for the CS treatment, in which a higher *1-FFT* expression than the 8% SUC treatment at 24 DAT (Fig. 7A) contrasted with lower levels of total FOS accumulation (Fig. 3A). Likewise, the high *1-FFT* expression levels detected in stems of PEG-Lo-treated *A. tequilana* plantlets at 42 DAT (Fig. 8A) did not translate into higher total FOS (Fig. 3A) or the biosynthesis of higher DP FOS (see Appendix S2 in Supporting Information).

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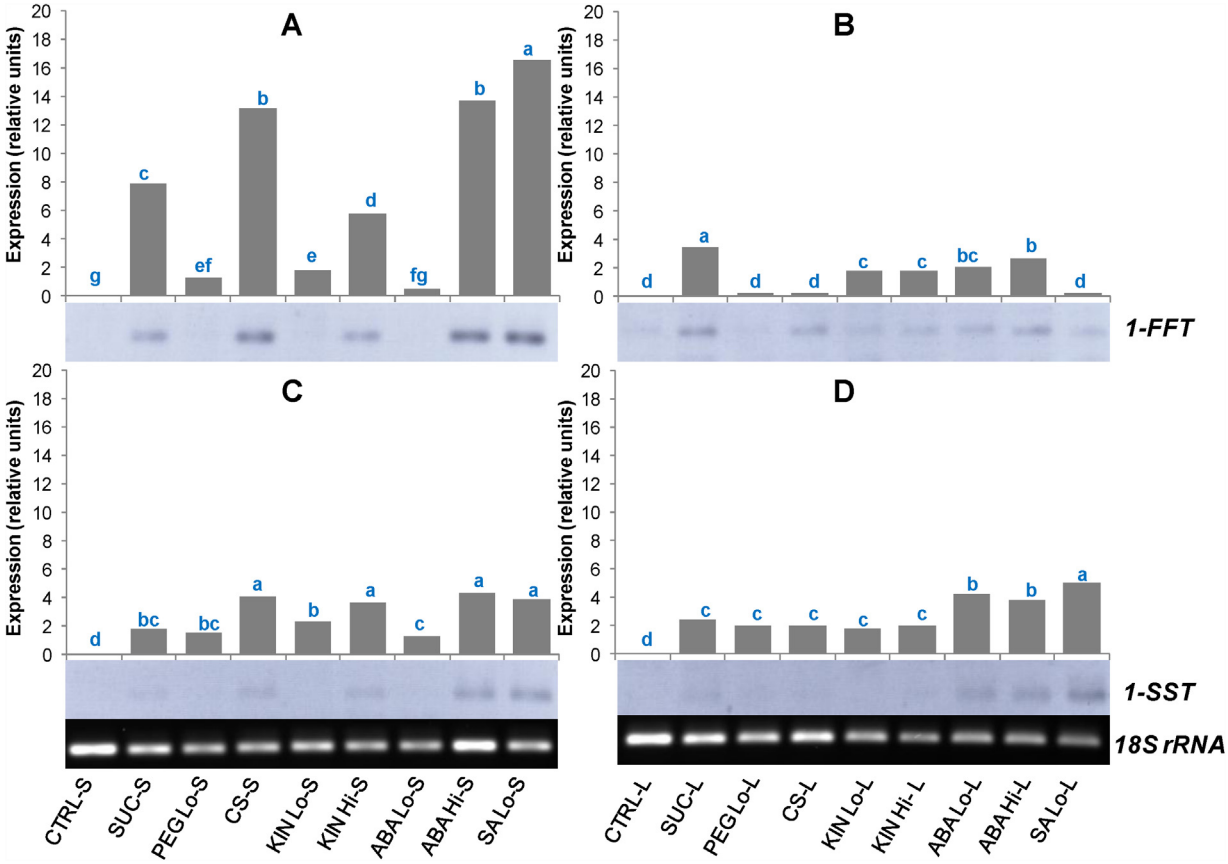

Fig. 7. Gene expression levels of *1-FFT* in (A) stem and (B) leaf tissue and *1-SST* in (C) stem and (D) leaf tissue of *A. tequilana* plantlets grown *in vitro* at 24 DAT. Expression was determined by semi-quantitative RT-PCR. The mean expression of these genes in relative units is shown. Different letters over the bars represent statistical difference at $P < 0.01$. Amplification of the constitutively expressed *rRNA 18S* gene, was used as loading control. Treatments included the application of cold stress (CS: 10 d at 0 °C + 14 or 32 d at 25 °C) or the addition to the medium of 8% sucrose (SUC), polyethylene glycol 10,000 (5% [PEG-Lo]), kinetin (0.5 mg l^{-1} [KIN-Lo] or 1 mg l^{-1} [KIN-Hi]), abscisic acid (10 μM [ABA-Lo] or 50 μM [ABA-Hi]) or salicylic acid (1 mM [SA-Lo]). Untreated controls are represented as CTRL.

In *A. inaequidens*, the relatively high expression levels of the *1-SST* and *1-FFT* genes detected at 24 DAT in leaves and stems of plantlets treated with ABA-Lo and SA-Lo (Fig. 9A and C) coincided both with total FOS accumulation (Fig. 4A) and the synthesis of higher DP FOS (Figs. 2A and 6B and Appendix S8 in Supporting Information). The weak MeJA-Hi-induced expression of these genes in stems and leaves after 24 DAT (Fig. 9A–D) was also congruent with the low level accumulation of FOS in tissues of MeJA-Hi-treated *A. inaequidens* plantlets only (Fig. 4A and B).

This contrasted with other treatments, in which the correlation between gene expression levels and FOS accumulation was not that straightforward. Thus, the significantly higher expression levels of both genes observed in stems and leaves of SUC-treated *A. inaequidens* plantlets 24 DAT (Fig. 9A, C and D) did not coincide with the rather low accumulation of total FOS (Fig. 4A and B) and higher DP FOS (Figs. 2A and 6A) observed. However, the higher expression levels at 24 DAT may pave the way for the increased FOS levels at 42 DAT (Figs. 2B, 4A, B and 6A). A consistent pattern was observed at 42 DAT, since the only close relationship between *1-SST* and *1-FFT* gene expression levels (Fig. 10) and FOS accumulation (Figs. 2B and 4) was observed in *A. inaequidens* plantlets treated with SA-Lo and MeJA-Hi (in stems only). The discrepancy between *1-SST* and *1-FFT* gene expression levels (Fig. 10) and FOS accumulation (Figs. 2B and 4A and B) was more evident in PEG-Hi- (stems), ABA-Lo- (leaves and stems) and MeJA-Hi-treated (leaves) plantlets.

Discussion

This study evaluated the effect that cold stress and the exogenous application of kinetin and various elicitors associated with (a)biotic stress had on FOS levels in *A. tequilana* and *A. inaequidens*. A significant difference between the two species is that the *A. tequilana* plants are mostly semi-domesticated elite plants that have been reproduced asexually by means of ramets, rhizomatous shoots or *in vitro* propagation, whereas *A. inaequidens* is collected in the wild (Valenzuela-Zapata et al., 2011). Differences were observed in the way the FOS composition of the two species is modified in leaves and/or stems in response to diverse treatments. In *A. tequilana*, induced fructan accumulation occurred predominantly in stems, whereas in *A. inaequidens* fructans accumulated both in stems and leaves, although the levels in the latter tissues were lower (compare Fig. 4A with B). Both species also responded differently to certain treatments. Fructan accumulation was not induced at all or at very weak levels in *A. inaequidens* plantlets subjected to CS, or treated with KIN, whereas it was more sensitive to ABA (*i.e.* it responded better to ABA-Lo than to ABA-Hi) and MeJA (*i.e.* MeJA-Hi was the best treatment to induce FOS accumulation) but resistant to the toxic effects of SA-Hi (at least at 24 DAT). On the other hand, and except for the CS and ABA-Lo treatments, FOS accumulation increased with time in both species, being highest at 42 DAT. Also, as shown in Figs. 1–6 and Appendix S2–S11, the amount of higher DP FOS generally increased in comparison to lower DP FOS in plants of both species sampled at 42 DAT, but more prominently in *A. tequilana*. The accumulation of low DP FOS was predominant in leaf tissue whereas higher DP FOS was predominant in stem tissue. The only exception was the MeJA-Hi treatment in *A. inaequidens*, which accumulated high DP FOS in leaves (Figs. 2B, 4B, 10B, D and Appendix S11).

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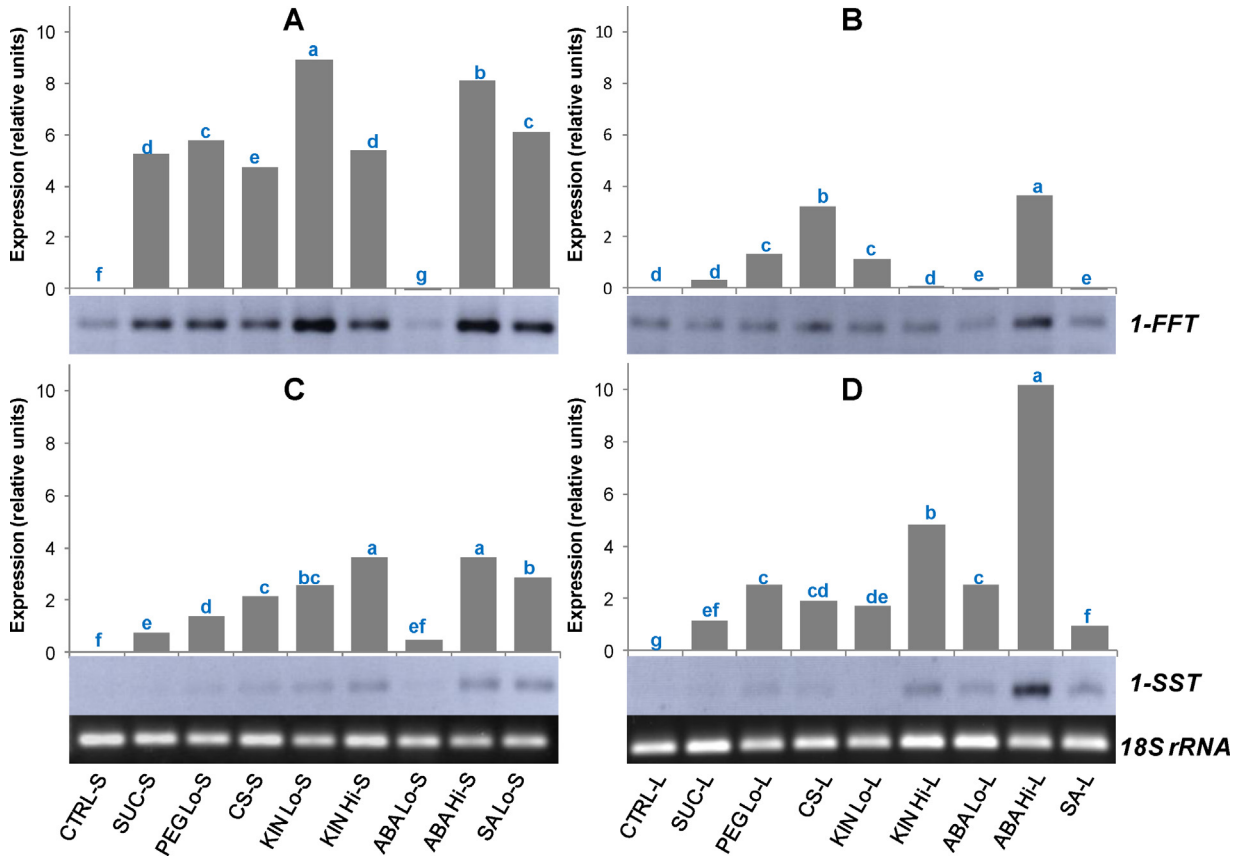

Fig. 8. Gene expression levels of *1-FFT* in (A) stem and (B) leaf tissue and *1-SST* in (C) stem and (D) leaf tissue of *A. tequilana* plantlets grown *in vitro* at 42 DAT. Different letters over the bars represent statistical difference at $P < 0.01$. Amplification of the constitutively expressed *rRNA 18S* gene, was used as loading control. The treatments are the same as those described in Fig. 7.

Sucrose and SA-Lo induced similar patterns of FOS accumulation in the stems of both species (Figs. 1–4). A time-dependent increase in FOS was also observed in leaves of both species, although it was more pronounced in *A. inaequidens* (Figs. 1B–4B). The results obtained in SUC treatments were consistent with previous reports describing a positive effect of Suc on fructan storage (Pollock and Cairns, 1991) and the fructan-synthesizing enzymes 1-FFT and 1-SST in various fructan accumulating species (Martinez-Noël et al., 2001; Van den Ende et al., 2006). This was due to the fact that the exogenous application of 8% Suc in the SUC treatment exceeded the threshold Suc concentration normally present in the MS media (3% Suc), required for the induced synthesis of fructans, which occurs through a cascade of events (Martinez-Noël et al., 2009; Ritsema et al., 2009; Joudi et al., 2012). Likewise, the potent inducing capacity of the SA treatment, at least at 1 mM, was also in agreement with several studies showing the positive effect that the exogenous application of this compound has on photosynthesis (Fariduddin et al., 2003; Khodary, 2004; Poór et al., 2011; Rivas San-Vicente and Plasencia, 2011), proteins and enzymes involved in plant carbohydrate/energy metabolism (Chan et al., 2008; Tarchevsky et al., 2010), particularly on sucrose phosphate synthase (SPS), the major enzyme involved in sucrose synthesis (Dong et al., 2011) and on favoring the accumulation of soluble sugars, especially of non-reducing sugars, in storage tissues such as roots (Dong et al., 2011). Data from wheat plants have suggested that Suc-elicited fructosyltransferase induction might involve the participation of protein kinases and phosphatases together with calcium and calcium-dependent kinases (Martinez-Noël et al., 2001, 2006).

Regarding SA, the effect on fructan levels observed at a concentration of 1 mM, which is lethal when applied to some other species (*i.e.* tomato [Szepesi et al., 2009; Gémes et al., 2011] and *Arabidopsis* [Borsani et al., 2001; Cao et al., 2009]) indicated that the *Agave* species tested are resistant to this phytotoxic phenolic compound. Toxicity is believed to be associated with an extensive oxidative damage produced when SA is applied at relatively high (≥0.1 mM) concentrations (Rao et al., 1997). This resistance was particularly evident in *A. inaequidens*, which was able to accumulate high levels of fructans, mostly in leaves at 24 DAT, with 20 mM SA (Fig. 2A). The SA-resistance observed in this wild species might have been a consequence of the necessary acclimatization to the dry conditions that these plants encounter in their natural habitat, which conferred them with an increased ability to better stand the oxidative stress associated with high SA levels. The unique responsiveness to MeJA shown by *A. inaequidens*, in both tissues and with the higher concentration employed, could be also explained in terms of drought-stress-acclimatization, considering that jasmonic acid (JA), in addition to ABA and SA, has been shown to be involved in drought stress resistance or drought survival, perhaps through the regulation of ascorbate and glutathione metabolism (Wasternack and Parthier, 1997; Shinozaki and Yamaguchi-Shinozaki, 2007; Ge et al., 2010; Shan and Liang, 2010; Brossa et al., 2011).

The low levels of *1-FFT* and *1-SST* expression observed in SUC-treated *A. inaequidens* at 42 DAT, which contrasted with its very strong expression at 24 DAT (Figs. 9 and 10), suggests that Suc-induced accumulation of FOS observed in stems of this species at 42 DAT (Figs. 2B and 6A) is the result of enzymes other than 1-FFT, such as fructan:fructan 6G-fructosyltransferase (6G-FFT) and sucrose:fructan 6-fructosyltransferase (6-SFT) (Cortés-Romero et al., 2012). This could also be said with regard to the MeJA-Hi treatment in stems. Another explanation could be that these treatments

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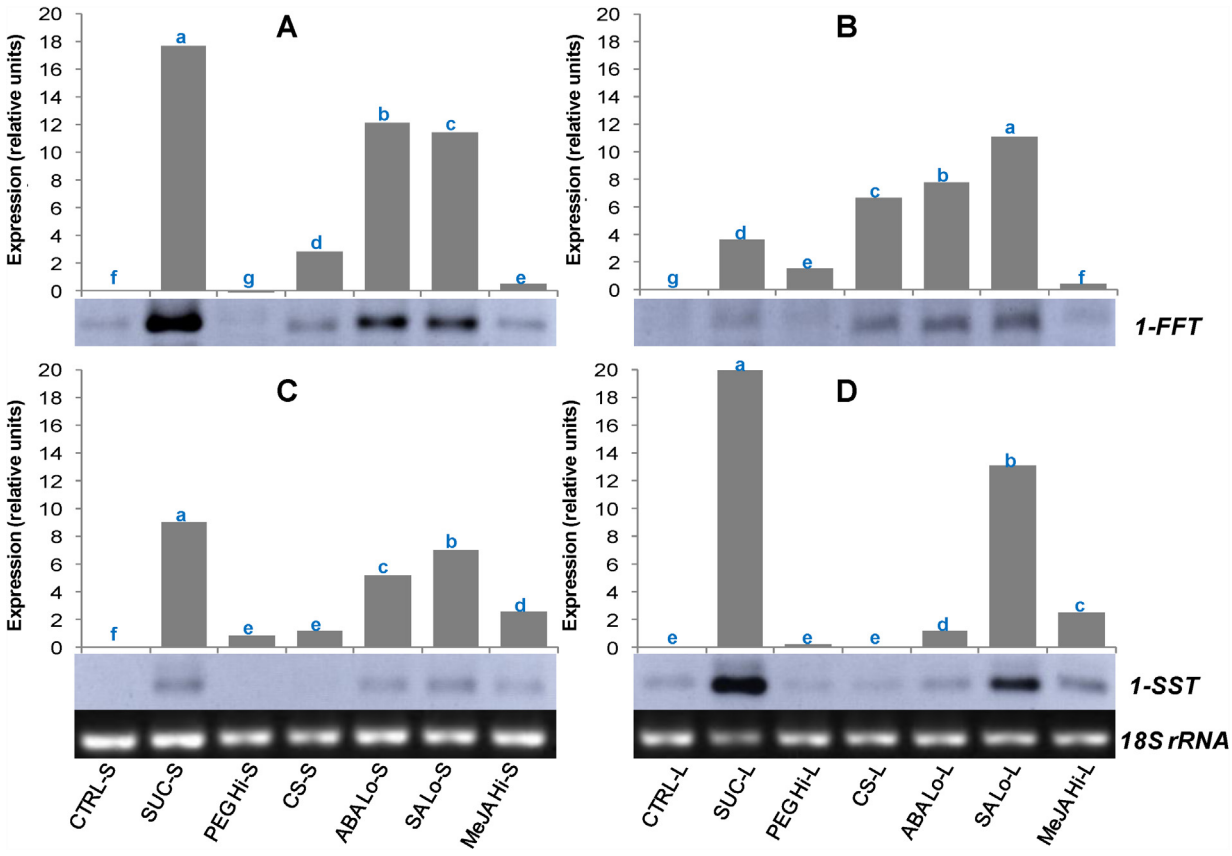

Fig. 9. Gene expression levels of *1-FFT* in (A) stem and (B) leaf tissue and *1-SST* in (C) stem and (D) leaf tissue of *A. inaequidens* plantlets grown *in vitro* at 24 DAT. Different letters over the bars represent statistical difference at $P < 0.01$. Amplification of the constitutively expressed *rRNA 18S* gene, was used as loading control. Treatments included the application of cold stress (CS: 10 d at 0 °C + 14 or 32 d at 25 °C) or the addition to the medium of 8% sucrose (SUC), polyethylene glycol 10,000 (10% [PEG-Hi]), abscisic acid (10 μM [ABA-Lo]), salicylic acid (1 mM [SA-Lo]), or methyl jasmonate (200 μM [MeJA-Hi]). Untreated controls are represented as CTRL.

repressed the activity of fructan exohydrolases (FEHs) involved in FOS degradation. Such a scenario is supported by experimental evidence showing that most FEHs are inhibited by Suc at the level of enzyme activity (Verhaest et al., 2007). In contrast, the known induction of FEHs by cold exposure in fructan accumulating species such as *Vernonia herbacea* (Portes et al., 2008) and chicory (Michiels et al., 2004) could explain the gradual reduction in FOS levels observed in *A. tequilana* plantlets once they were released from the imposed stress (Figs. 1 and 3 and Appendix S2 in Supporting Information). However, in all other treatments, a close correlation between low and high FOS accumulation (Figs. 1–6) and the expression of genes coding for fructan synthesizing enzymes, particularly 1-FFT in stem tissue (Figs. 7–10), suggests that this enzyme could be responsible of catalyzing most of the induced synthesis of FOS in both *A. tequilana* and *A. inaequidens*. This is in accordance with the earlier description of the positive influence that SUC exerts on the levels of *1-FFT* and *1-SST* expression and activity, which is followed by increased fructan content (Van den Ende et al., 2006). The inductive effect of ABA can be attributed to the intimate cross-talk of ABA and sugar signaling pathways (Dekkers et al., 2008; Bolouri Moghaddam and Van den Ende, 2012). In addition, the observed induction by ABA and PEG could be explained on the basis of the cumulative evidence showing the role played by fructans in the amelioration of salt and/or drought stress (Pilon-Smits et al., 1995; 1999; Valluru and Van den Ende, 2008; Dong et al., 2011; Garcia et al., 2011) together with the reported inducing effect of drought conditions on fructan accumulation and the 1-SST enzyme (De Roover et al., 2000). On the other hand, the observed accumulation of low DP FOS in the treatment with high concentration of ABA in stem and leaf tissue of *A. inaequidens* was probably due to the stimulation of FEHs and invertases (Yang et al., 2004). SA, as mentioned, has a positive effect on non-reducing sugar accumulation and synthesis of SUC synthesizing enzymes such as SPS, and it is likely that this effect may extend to fructan synthesizing enzymes too, as suggested by the results shown in Figs. 7–10. In contrast, the mechanism(s) behind the inductive effect of MeJA (in *A. inaequidens*) and KIN (in *A. tequilana*) on the fructan biosynthetic genes examined in this study remain to be determined. However, evidence is available showing that sugars and JA have a connection in the signal cascades in the systemic defense response after herbivore or pest attraction such as ants and other predatory insects (Heil et al., 2012). Moreover, JA can regulate sink source relationships in mycorrhizal plants by controlling key genes in sugar metabolism (Tejeda-Sartorius et al., 2008). Zeatin, a cytokinin structurally related to KIN, is capable of inducing the synthesis of fructans in calli of *S. officinale* (Haaß et al., 1991). This may be related to the fact that zeatin and BAP were found to be the optimal phytohormones for fructan induction, which was correlated with growth rate and organ differentiation in tissue culture. In fact, fructan metabolism seems to be under phytohormonal control to some degree (Haaß et al., 1991); the latter is coincident with our experiments showing fructan accumulation in agave plants treated with both ABA and KIN.

In conclusion, this work provides evidence that SA, MeJA and ABA play an important role in the accumulation of FOS in two *Agave* species. The effect was particularly strong in both species when 1 mM SA was applied. In general, the observed accumulation of high DP FOS in stems, a storage tissue in agaves, correlated with a higher expression of the *1-FFT* gene, whereas in leaf tissue, the effect was inverted, since the predominant expression of the *1-SST* gene was

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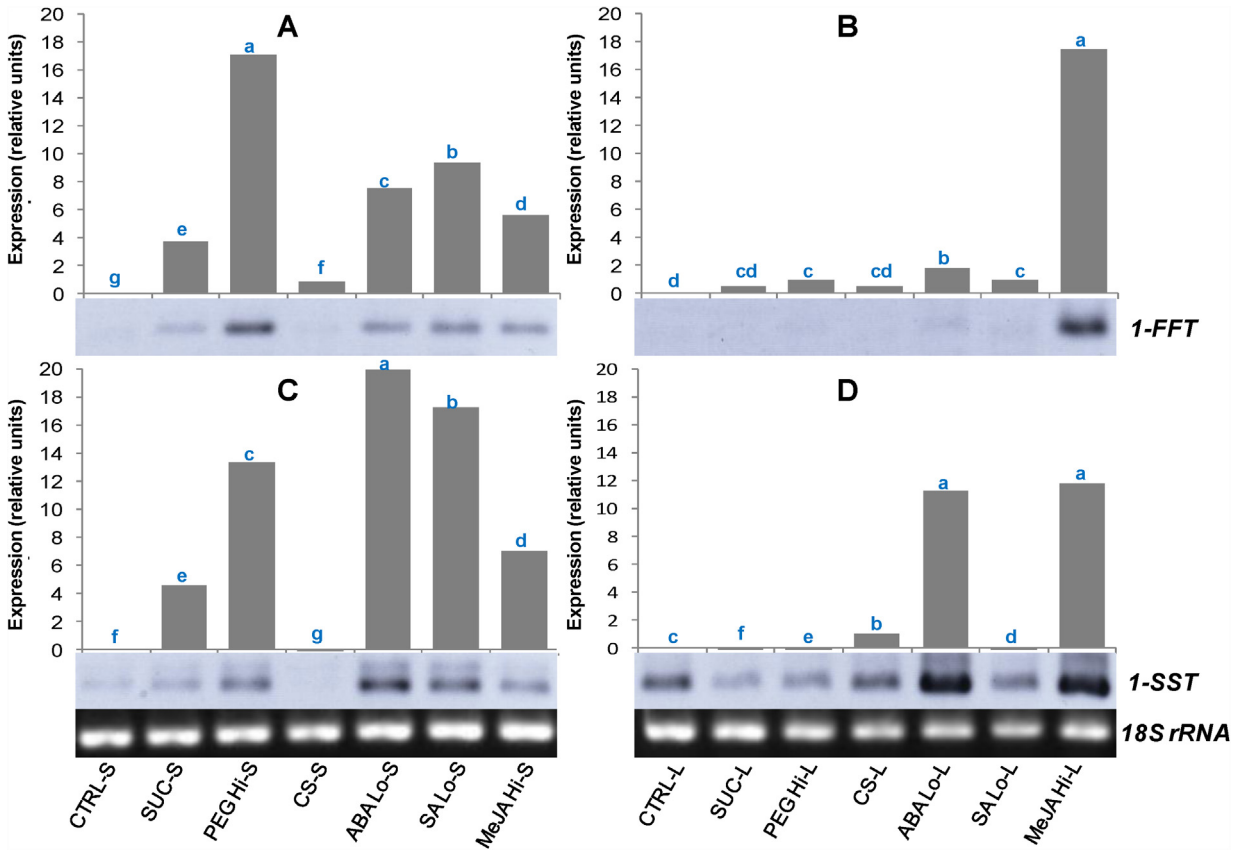

Fig. 10. Gene expression levels of *1-FFT* in (A) stem and (B) leaf tissue and *1-SST* in (C) stem and (D) leaf tissue of *A. inaequidens* plantlets grown *in vitro* at 42 DAT. Different letters over the bars represent statistical difference at $P < 0.01$. Amplification of the constitutively expressed *rRNA 18S* gene, was used as loading control. The treatments are the same as those described in Fig. 9.

consistent with the accumulation of low DP FOS. Importantly, the control treatment showed little or no accumulation of FOS, a result coincident with the low expression of the *1-SST* and *1-FFT* genes. Moreover, the results of this study show that this *in vitro* model is useful for the evaluation of inducers-repressors of genes involved in fructan biosynthesis in agave plants, which normally complete their life cycle in a period ranging from 8 to 12 years. The role of FEHs in the mobilization and redistribution of FOS in response to different (a)biotic stimuli remains to be elucidated.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2013.08.002.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. J Mol Biol 1990;215:403–10.
Anderson K, Li SC, Li YT. Diphenylamine–aniline–phosphoric acid reagent, a versatile spray reagent for revealing glycoconjugates on thin-layer chromatography plates. Anal Biochem 2000;287:337–9.
Bolouri Moghaddam MR, Van den Ende W. Sugars and plant innate immunity. J Exp Bot 2012;63:3989–98.
Borsani O, Valpuesta V, Botella MA. Evidence for a role of salicylic acid in the oxidative damage generated by NaCl and osmotic stress in Arabidopsis seedlings. Plant Physiol 2001;126:1024–30.
Brossa R, López-Carbonell M, Jubany-Marí T, Alegre L. Interplay between abscisic acid and jasmonic acid and its role in water-oxidative stress in wild-type, ABA-deficient, JA-deficient, and ascorbate-deficient Arabidopsis plants. J Plant Growth Regul 2011;30:322–33.
Cao Y, Zhang ZW, Xue LW, Du JB, Shang J, Xu F, et al. Lack of salicylic acid in Arabidopsis protects plants against moderate salt stress. Z Naturforsch 2009;64c:231–8.
Chan Z, Wang Q, Xu X, Meng X, Qin G, Li B, et al. Functions of defense-related proteins and dehydrogenases in resistance response induced by salicylic acid in sweet cherry fruits at different maturity stages. Proteomics 2008;8:4791–807.
Cortés-Romero C, Martínez-Hernández A, Mellado-Mojica E, López MG, Simpson J. Molecular and functional characterization of novel fructosyltransferases and invertases from *Agave tequilana*. PLoS ONE 2012;7:e35878.
Darwen CE, John P. Localization of the enzymes of fructan metabolism in vacuoles isolated by a mechanical method from tubers of Jerusalem artichoke (*Helianthus tuberosus* L.). Plant Physiol 1989;89:658–63.
De Coninck B, Le Roy K, Francis I, Clerens S, Vergauwen R, Halliday AM, et al. Arabidopsis AtcwINV3 and 6 are not invertases but are fructan exohydrolases (FEHs) with different substrate specificities. Plant Cell Environ 2005;28:432–43.
De Roover J, Vandenbranden K, Van Laere A, Van den Ende W. Drought induces fructan synthesis and 1-SST (sucrose: sucrose fructosyltransferase) in roots and leaves of chicory seedlings (*Cichorium intybus* L.). Planta 2000;210:808–14.
Dekkers BJ, Schuurmans JA, Smeekens SC. Interaction between sugar and abscisic acid signalling during early seedling development in Arabidopsis. Plant Mol Biol 2008;67:151–67.
Dong C, Wang X, Shang Q. Salicylic acid regulates sugar metabolism that confers tolerance to salinity stress in cucumber seedlings. Sci Hort 2011;129:629–36.
Fariduddin Q, Hayat S, Ahmad A. Salicylic acid influences net photosynthetic rate, carboxylation efficiency, nitrate reductase activity and seed yield in *Brassica juncea*. Photosynthetica 2003;41:281–4.
Frehner M, Keller F, Wiemken A. Localization of fructan metabolism in vacuoles isolated from protoplasts of Jerusalem artichoke tubers. J Plant Physiol 1984;116:197–208.

Please cite this article in press as: Suárez-González EM, et al. Expression of the 1-SST and 1-FFT genes and consequent fructan accumulation in *Agave tequilana* and *A. inaequidens* is differentially induced by diverse (a)biotic-stress related elicitors. J Plant Physiol (2013), http://dx.doi.org/10.1016/j.jplph.2013.08.002

Garcia PMA, Asega AF, Silva EA, Carvalho MAM. Effect of drought and re-watering on fructan metabolism in *Vernonia herbacea* (Vell) Rusby. Plant Physiol Biochem 2011;49:664–70.
García-Mendoza AJ. Los agaves de México. Ciencias 2007;87:14–23.
Ge Y, Zhang L, Li F, Chen Z, Wang C, Yao Y, et al. Relationship between jasmonic acid accumulation and senescence in drought-stress. Afr J Agric Res 2010;5:1978–83.
Gémes K, Poór P, Horváth E, Kolbert Z, Szopkó D, Szepesi A, et al. Cross-talk between salicylic acid and NaCl-generated reactive oxygen species and nitric oxide in tomato during acclimation to high salinity. Physiol Plant 2011;142:179–92.
Godoy-Hernández G, Loyola-Vargas VM. Effect of acetylsalicylic acid in secondary metabolism of *Catharanthus roseus* tumor suspension cultures. Plant Cell Rep 1997;16:287–90.
Gomez E, Tuohy KM, Gibson GR, Klinder A, Costabile A. In vitro evaluation of the fermentation properties and potential prebiotic activity of Agave fructans. J Appl Microbiol 2010;108:214–21.
Haaß D, Abou-Mandour AA, Blaschek W, Franz G, Czygan FC. The influence of phytohormones on growth, organ differentiation and fructan production in callus of *Symphytum officinale L.* Plant Cell Rep 1991;10:421–4.
Hayat S, Ahmad A. Salicylic Acid, A Plant Hormone. Springer, Dordrecht: The Netherlands; 2007.
Heil M, Ibarra-Laclette E, Adame-Álvarez RM, Martinez O, Ramirez-Chávez E, Molina-Torres J, et al. How plants sense wounds: damaged-self recognition is based on plant-derived elicitors and induces octadecanoid signaling. PLoS ONE 2012;7:e30537.
Hisano H, Kanazawa A, Yoshida M, Humphreys MO, Iizuka M, Kitamura K, et al. Coordinated expression of functionally diverse fructosyltransferase genes is associated with fructan accumulation in response to low temperature in perennial ryegrass. New Phytol 2008;178:766–80.
Janda T, Szalai G, Tari I, Paldi E. Hydroponic treatment with salicylic acid decreases the effects of chilling injury in maize (*Zea mays* L.) plants. Planta 1999;208:175–80.
Joudi M, Ahmadi A, Mohamadi V, Abbasi A, Vergauwen R, Mohammadi H, et al. Comparison of fructan dynamics in two wheat cultivars with different capacities of accumulation and remobilization under drought stress. Physiol Plant 2012;144:1–12.
Kawakami A, Sato Y, Yoshida M. Genetic engineering of rice capable of synthesizing fructans and enhancing chilling tolerance. J Exp Bot 2008;59:793–802.
Kerepesi I, Galiba G. Osmotic and salt stress-induced alteration in soluble carbohydrate content in wheat seedlings. Crop Sci 2000;40:482–7.
Khodary SEA. Effect of salicylic acid on the growth, photosynthesis and carbohydrate metabolism in salt stressed maize plants. Int J Agric Biol 2004;6:5–8.
Knipp G, Honermeier B. Effect of water stress on proline accumulation of genetically modified potatoes (*Solanum tuberosum* L.) generating fructans. J Plant Physiol 2006;163:392–7.
Kusch U, Greiner S, Steininger H, Meyer AD, Corbière-Divialle H, Harms K, et al. Dissecting the regulation of fructan metabolism in chicory (*Cichorium intybus*) hairy roots. New Phytol 2009;184:127–40.
Landjeva S, Neumann K, Lohwasser U, Börner A. Molecular mapping of genomic regions associated with wheat seedling growth under osmotic stress. Biol Plant 2008;52:259–66.
Li HJ, Yang AF, Zhang XC, Gao F, Zhang JR. Improving freezing tolerance of transgenic tobacco expressing-sucrose sucrose 1-fructosyltransferase gene from *Lactuca sativa*. Plant Cell Tiss Org Cult 2007;89:37–48.
Livingston DP, Hincha DK, Heyer AG. Fructan and its relationship to abiotic stress tolerance in plants. Cell Mol Life Sci 2009;66:2007–23.
Mancilla-Margalli NA, López MG. Water soluble carbohydrates and fructans structure patterns from *Agave* and *Dasylirion* species. J Agric Food Chem 2006;54:7832–9.
Martinez-Noël GM, Tognetti JA, Pontis HG. Protein kinase and phosphatase activities are involved in fructan synthesis initiation mediated by sugars. Planta 2001;213:640–6.
Martinez-Noël GMA, Tognetti JA, Nagaraj VJ, Wiemken A, Pontis HG. Calcium is essential for fructan synthesis induction mediated by sucrose in wheat. Planta 2006;225:183–91.
Martinez-Noël GMA, Tognetti JA, Salerno GL, Wiemkem A, Pontis HG. Protein phosphatase activity and sucrose-mediated induction of fructan synthesis in wheat. Planta 2009;230:1071–9.
Michiels A, Van Laere A, Van den Ende W, Tucker M. Expression analysis of a chicory fructan 1-exohydrolase gene reveals complex regulation by cold. J Exp Bot 2004;55:1325–33.
Montgomery DC. Diseño y Análisis de Experimentos. México: Limusa Wiley; 2004.
Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 1962;15:473–97.
Nobel PS. Environmental influences on CO_2 uptake by Agaves, CAM plants with high productivities. Econ Bot 1990;44:488–502.
Onofre J, Solano JL, Avonce N, Iturriaga G. Root specific expression of ahybadh4 gene promoter in transgenic *Arabidopsis thaliana* plants. Rev Fitotec Mex 2003;26:133–40.
Peshev D, Vergauwen R, Moglia A, Hideg E, Van den Ende W. Towards understanding vacuolar antioxidant mechanisms: a role for fructans. J Exp Bot 2013;64:1025–38.
Pilon-Smits EAH, Ebskamp MJM, Paul MJ, Jeuken MJW, Weisbeek PJ, Smeekens SCM. Improved performance of transgenic fructan-accumulating tobacco under drought stress. Plant Physiol 1995;107:125–30.
Pilon-Smits EAH, Terry N, Sears T, Van Dun K. Enhanced drought resistance in fructan-producing sugar beet. Plant Physiol Biochem 1999;37:313–7.
Pimienta-Barrios E, Zañudo-Hernández J, Nobel PS, García-Galindo J. Respuesta fisiológica a factores ambientales del agave azul (*Agave tequilana* Weber). Scientia-CUCBA 2005;7:85–97.
Pollock CJ, Cairns AJ. Fructan metabolism in grasses and cereals. Annu Rev Plant Physiol Plant Mol Biol 1991;42:77–101.
Pollock CJ, Eagles CF, Sims IM. Effect of photoperiod and irradiance changes upon development of freezing tolerance and accumulation of soluble carbohydrate in seedlings of *Lolium perenne* grown at 2 °C. Ann Bot 1988;62:95–100.
Poór P, Gémes K, Horváth F, Szepesi A, Simon ML, Tari I. Salicylic acid treatment via the rooting medium interferes with stomatal response, CO_2 fixation rate and carbohydrate metabolism in tomato, and decreases harmful effects of subsequent salt stress. Plant Biol 2011;13:105–14.
Portes MT, de Cássia R, Figueiredo-Ribeiro L, de Carvalho MAM. Low temperature and defoliation affect fructan-metabolizing enzymes in different regions of the rhizophores of *Vernonia herbacea*. J Plant Physiol 2008;165:1572–81.
Rao MV, Paliyath G, Ormrod DP, Murr DP, Watkins CB. Influence of salicylic acid on H_2O_2 production, oxidative stress, and H_2O_2-metabolizing enzymes (salicylic acid-mediated oxidative damage requires H_2O_2). Plant Physiol 1997;115:137–49.
Ritsema T, Brodmann D, Diks SH, Bos CL, Nagaraj VJ, Pieterse CMJ, et al. Are small GTPases signal in sugar-mediated induction of fructan biosynthesis? PLoS ONE 2009;4:e6605.
Rivas San-Vicente M, Plasencia J. Salicylic acid beyond defence: its role in plant growth and development. J Exp Bot 2011;62:3321–38.
Shan C, Liang Z. Jasmonic acid regulates ascorbate and glutathione metabolism in *Agropyron cristatum* leaves under water stress. Plant Sci 2010;178:130–9.
Shinozaki K, Yamaguchi-Shinozaki K. Gene networks involved in drought stress response and tolerance. J Exp Bot 2007;58:221–7.
Silvakumar G, Paek KY. Methyl jasmonate induce enhanced production of soluble biophenols in *Panax ginseng* adventitious roots from commercial scale bioreactors. Chem Nat Comp 2005;41:669–73.
Singh B, Usha K. Salicylic acid induced physiological and biochemical changes in wheat seedlings under water stress. Plant Growth Reg 2003;39:137–41.
Statpoint, Inc STATGRAPHICS Centurion XV 2007; Version 15.2.06.
Szepesi A, Csiszára J, Gémesa K, Horváth E, Horváth F, Simon ML, et al. Salicylic acid improves acclimation to salt stress by stimulating abscisic aldehyde oxidase activity and abscisic acid accumulation, and increases Na^+ content in leaves without toxicity symptoms in *Solanum lycopersicum* L. J Plant Physiol 2009;166:914–25.
Tarchevsky IA, Yakovleva VG, Egorova AM. Proteomic analysis of salicylate-induced proteins of pea (*Pisum sativum* L.) leaves. Biochemistry (Mosc) 2010;75:590–7.
Tejeda-Sartorius M, Martínez de la Vega O, Délano-Frier JP. Jasmonic acid influences mycorrhizal colonization in tomato plants by modifying the expression of genes involved in carbohydrate partitioning. Physiol Plant 2008;133:339–53.
Turkan I, Bor M, Zdemir F, Koca H. Differential responses of lipid peroxidation and antioxidants in the leaves of drought-tolerant *P. acutifolius* and drought-sensitive *P. vulgaris* subjected to polyethylene glycol mediated water stress. Plant Sci 2005;168:223–31.
Urías-Silvas JE, Cani PD, Delmeé E, Neyrinck AM, López MG, Delzenne NM. Physiological effect of dietary fructans extracted from *Agave tequilana* Gto and *Dasylirion* spp. Br J Nutr 2008;99:254–61.
Valenzuela-Sánchez KK, Juárez-Hernández RE, Cruz-Hernández A, Olalde-Portugal V, Valverde ME, Paredes-López O. Plant regeneration of *Agave tequilana* by indirect organogenesis. In Vitro Cell Dev Biol Plant 2006;42:336–40.
Valenzuela-Zapata AG, Lopez-Muraira I, Gaytán SM. Traditional knowledge, *Agave inaequidens* (Koch) conservation, and the charro lariat artisans of San Miguel Cuyutlán, México. Soc Ethnobiol 2011;2:72–80.
Valluru R, Van den Ende W. Plant fructans in stress environments: emerging concepts and future prospects. J Exp Bot 2008;59:2905–16.
Valluru R, Lammens W, Claupein W, Van den Ende W. Freezing tolerance by vesicle-mediated fructan transport. Trends Plant Sci 2008;13:409–14.
Van den Ende W, Michiels A, De Roover J, Verhaert P, Van Laere A. Cloning and functional analysis of chicory root fructan 1-exohydrolase I (1-FEH): a vacuolar enzyme derived from a cell wall invertase ancestor? Mass fingerprint of the 1-FEH I enzyme. Plant J 2000;24:447–56.
Van den Ende W, Michiels A, Van Wonterghem D, Clerens SP, De Roover J, Van Laere AJ. Defoliation induces fructan 1-exohydrolase II in Witloof chicory roots. Cloning and purification of two isoforms, fructan 1-exohydrolase IIa and fructan 1-exohydrolase IIb. Mass fingerprint of the fructan 1-exohydrolase II enzymes. Plant Physiol 2001;126:1186–95.
Van den Ende W, Clerens S, Vergauwen R, Van Riet L, Van Laere A, Yoshida M, et al. Fructan 1-exohydrolases, Beta-(2,1)-trimmers during graminan biosynthesis in stems of wheat? Purification, characterization, mass mapping and cloning of two fructan 1-exohydrolase isoforms. Plant Physiol 2003a;131:621–31.
Van den Ende W, De Coninck B, Clerens S, Vergauwen R, Van Laere A. Unexpected presence of fructan 6-exohydrolases (6-FEHs) in non-fructan plants: characterization, cloning, mass mapping and functional analysis of a novel cell-wall invertase-like specific 6-FEH from sugar beet (*Beta vulgaris* L.). Plant J 2003b;36:697–710.
Van den Ende W, Clerens S, Vergauwen R, Boogaerts D, Le Roy K, Arckens L, et al. Cloning and functional analysis of a high DP fructan: fructan 1-fructosyl transferase from *Echinops ritro* (Asteraceae): comparison of the native and recombinant enzymes. J Exp Bot 2006;57:775–89.
Van Riet L, Nagaraj V, Van den Ende W, Clerens S, Wiemken A, Van Laere A. Purification, cloning and functional characterization of a fructan 6-exohydrolase from wheat (*Triticum aestivum* L.). J Exp Bot 2006;57:213–23.

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Verhaest M, Lammens W, Le Roy K, De Ranter CJ, Van Laere A, Rabijns A, et al. Insights into the fine architecture of the active site of chicory fructan 1-exohydrolase: 1-kestose as substrate vs. sucrose as inhibitor. New Phytol 2007;174:90–100.
Vijn I, Smeekens S. Fructan: more than a reserve carbohydrate. Plant Physiol 1999;120:351–9.
Wasternack C, Parthier B. Jasmonate-signalled plant gene expression. Trends Plant Sci 1997;2:302–7.
Wiemken A, Smeekens S. Fructan of the inulin neoseries is synthesized in transgenic chicory plants (*Chichorium intybus* L.) harbouring onion (*Allium cepa* L.) fructan: fructan 6G-fructosyltransferase. Plant J 1997;11:387–98.
Yang J, Zhang J, Wang Z, Zhu Q, Liu L. Activities of fructan- and sucrose-metabolizing enzymes in wheat stems subjected to water stress during grain filling. Planta 2004;220:331–43.

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