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Fructan reduction by downregulation of *1-SST* in guayule

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ABSTRACT

The natural rubber producing plant guayule (*Parthenium argentatum* Gray) stores carbohydrates mainly in the form of fructans, synthesized and stored in the same tissues at the same time as the rubber polymer, and a potential source of carbon for rubber biosynthesis. The first committed step to fructan synthesis is catalyzed by sucrose:sucrose-1-fructosyltransferase (1-SST), which was downregulated to test whether reduction of carbohydrate synthesis would divert carbon instead to rubber biosynthesis. Guayule leaf strips were transformed by *Agrobacterium*-mediated technology, and plants with downregulated *1-SST* were evaluated in the laboratory and greenhouse. The plant tissue fructan concentration was reduced significantly, and sucrose concentration increased, especially in root tissues of greenhouse-grown plants. However, increased natural rubber production did not result.

1. Introduction

Guayule, a woody desert shrub under cultivation as a source of domestic natural rubber, stores carbohydrates in the form of fructans levulin and inulin (Hassid et al., 1944; McRary and Traub 1944; Traub and Slattery, 1946a). Fructans are polymers consisting of fructose units with a terminal glucose residue. They serve as carbon reserves when the plant transitions from carbon utilization to carbon storage. Fructans are hydrolyzed when dormancy breaks to provide additional carbon for new growth. In guayule, most (50–75%) of the fructans are found in roots and stems, and are involved in maintenance of turgor under stress (Kelly and Van Staden, 1991). Fructan buildup during cold acclimation has been correlated with increased freezing tolerance (Eagles, 1967; Livingston et al., 2009; Pontis, 1989). Fructans are thought to act to protect plants against drought and cold stress through cellular membrane stabilization. Insertion of at least a part of the polysaccharide into the lipid headgroup region of the membrane may help prevent leakage when water is removed from the system, either during freezing or drought (Livingston et al., 2009).

In guayule, most fructan synthesis takes place at the onset of and during winter conditions (Appleton and van Staden, 1989; Kelly and Van Staden, 1991, 1993; Salvucci et al., 2010; Traub and Slattery, 1946a) parallel to natural rubber biosynthesis (Bonner, 1943; Bonner and Galston, 1947). In this so-called 'dormant' state, guayule plants are still quite photosynthetically active, fixing carbon at the same or greater rates than during growth periods (Salvucci et al., 2010), apparently due to lower levels of respiration (Paterson-Jones et al., 1990).

In guayule, sucrose is synthesized in the leaves, translocated to the stem, and converted to acetate which serves as the major source of carbon for rubber biosynthesis and resin production (Kelly and Van Staden, 1987, 1993). Evidence that carbohydrates can be depolymerized to provide carbon for rubber synthesis has been found in guayule (Kelly and Van Staden, 1991, 1993) and dandelion (Stolze et al., 2016). Regardless, since sucrose is the precursor to both metabolites, biosynthesis of natural rubber and fructans may compete for available carbon in the same plant tissues at the same time (Fig. 1).

The synthesis of fructan in plants is catalyzed by three different classes of enzymes: sucrose:sucrose-1-fructosyltransferase (EC 2.4.1.99) (1-SST), fructan:fructan 1-fructosyltransferase (EC 2.4.1.100) (1-FFT), and fructan exohydrolase (EC3.2.1.153) (1-FEH) (Edelman and Jeford, 1968). The 1-SST enzyme primarily catalyses the synthesis of 1-kestose from two molecules of sucrose, the first committed step to fructan biosynthesis. In this study, *1-SST* was downregulated by genetic transformation to determine if carbon flux could be deviated from carbohydrate production to natural rubber production in guayule.

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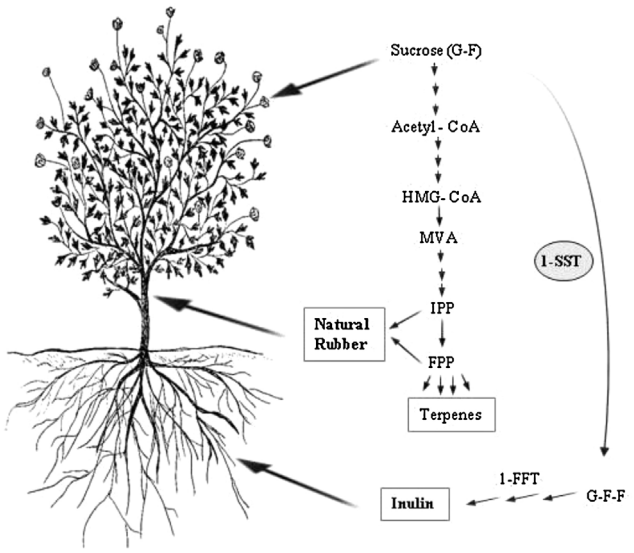

Fig. 1. Abbreviated carbon flux in guayule: Leaf photosynthetic products glucose (G) and fructose (F) combine to form sucrose (G + F). 1-SST catalyzes synthesis of sucrose + fructose, the first committed step to biosynthesis of inulin, primarily stored in roots. Alternatively, sucrose forms acetyl CoA, which targets carbon to natural rubber, stored mainly in stems, via the mevalonate pathway. HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A; MVA: mevalonate; IPP: isopentenyl pyrophosphate; FPP: farnesyl pyrophosphate; 1-SST: sucrose:sucrose 1-fructosyltransferase; 1-FFT: fructan:fructan 1-fructosyltransferase. Sketch by D. Lhamo, used with permission.

2. Methods and materials

2.1. Maintenance of guayule plants in vitro

Guayule line G7-11 was established as described previously (Castillon and Cornish, 2000). The G7-11 line is an earlier version of a line later released as AZ-2 (Ray et al., 1999). A shoot tip 15 mm or longer was excised and inoculated into a Magenta box containing 80 mL fresh ½ MS- I0.1 [half-strength MS medium (Murashige and Skoog, 1962) plus $CaCl_2$ (2.5 mM), $MgSO_4$ (2.0 mM) and IBA (0.1 mg/L) sucrose (15 g/L) and agar (8 g/L) at pH 5.8]. The cultures were maintained at 25 °C under cool-white fluorescent light (~50 µmol/m^2/s, 16/8-h day/night photoperiod). Tissue culture plants were propagated on a ca. 2–4 month basis throughout the study. Controls and *1-SST RNAi* plants were maintained under these conditions for tissue culture phenotype analysis.

2.2. Molecular cloning of partial guayule 1-SST gene and selection of the RNAi target

The sucrose:sucrose 1-fructosyl transferase (*1-SST*) mRNA sequence (DY813230.1) isolated from *Taraxacum officinale* (common dandelion) was used to search for the homologous sequence in the *Taraxacum koksaghyz* (*TKS*; Russian dandelion) EST database. A 734 bp EST sequence (GO668020.1) was the output of this search.

To clone the partial *1-SST* gene in guayule, the first 23 bp from this EST sequence was used as a forward primer and the last 26 bp was used as a reverse primer. The primer pair was used in a PCR of guayule genomic DNA with Promega Hot Start Taq Polymerase. A DNA fragment of > 0.7 Kb was amplified. This DNA fragment was cloned into pGEM-T vector and sequenced. A DNA sequence of 418 bp was selected as the RNAi target (Fig. 2).

2.3. Plasmid construction

Empty vector pND9 was constructed based on pPZP200 (Hajdukiewicz et al., 1994) which contains a 409 promoter (Rockhold et al., 2008) driven NPTII gene (Herrera-Estrella et al., 1983) with an Ocs terminator (De Greve et al., 1982), and a MtHP promoter (Xiao et al., 2005) driven GUSplus gene (Jefferson, 2001) with a potato Ubiquitin 3 terminator (Garbarino and Belknap, 1994). Plasmid pND9-*1-SSTi* (Fig. 3) was constructed by replacing the GUSplus gene with an inverted repeat of *1-SST* sequence. The repeat contained a reverse complimentary 418 bp in its 5′ end and the forward 418 bp in its 3'end (Fig. 2). The BAR gene (Christensen and Quail, 1996) of 552 bp was inserted into the middle of this inverted repeat. Plasmids pND9 and *pND9-1-SSTi* were used to transform *Agrobacterium* EHA101 (Hood et al., 1986) competent cells. The transformed *Agrobacterium* EHA101 harboring pND9 or *pND9-1-SSTi* were used to transform the guayule G7-11 plants.

Agrobacterium overnight cultures were prepared by inoculating 20 µL of a long-term glycerol stock into a 50 mL Falcon tube containing 5 mL LB medium plus rifampcin (40 mg/L) and spectinomycin (200 mg/L) with shaking at 200 rpm at 28 °C. The culture was then centrifuged for 15 min at 1600 × *g* at room temperature. The supernatant was discarded and the pellet was re-suspended in 25 mL of Inoculation Solution composed of 1/10MS salts plus BA (2 mg/L), NAA (0.5 mg/L), glucose (10 g), acetosyringone (200 µM), and pluronic F68 (0.05%) at pH 5.2.

2.4. Leaf tissue transformation

Leaf strip transformation followed Dong et al. (2006) with some modifications. Leaves were cut from *in vitro* grown guayule plants. Each leaf was placed in a Petri dish containing 5 mL of *Agrobacterium* suspension (containing the *pND9:1-SST RNAi* construct) with the adaxial side (upper side) facing up. Leaf strips were cut into 10 mm strips, blot-dried with sterile filter paper, and transferred one by one (not overlapping) to an empty Petri dish. The Petri dish was sealed with parafilm and placed in the dark. The co-cultivation was replaced by this co-desiccation step following Cheng et al. (2003). After 3 days in the dark, leaf strips were transferred to MS-B1T400 medium composed of MS medium plus $CaCl_2$ (2.5 mM), $MgSO_4$ (2.0 mM), BA (1 mg/L), NAA (0.25 mg/L), sucrose (30 g/L), Phytagel (3 g/L, Sigma-Aldrich, USA, Cat# P8169), and timentin (400 mg/L) (Cheng et al., 1998) was added for recovery at low light for 5 days. The leaf strips were then transferred to selection medium MS-B0.75TK30 (MS-B1T400) with reduced concentration of BA (0.75 mg/L), timentin (250 mg/L), and kanamycin (30 mg/L) for selection under low light.

Two weeks later, the leaf strips were transferred to MS-B0.75TK30 and subcultured every 2 weeks under high light until green shoots emerged. Green shoots 15 mm and longer were transferred to ½ MS-I0.1TKI0 (½ MS-10.1) with adjusted timentin (250 mg/L) and kanamycin (10 mg/L) levels, for rooting for 2–4 weeks. Rooted plantlets were transferred to half-strength MS medium for maintenance or transplanted into soil. These plantlets are referred to as tissue culture plants or TC plants hereafter.

2.5. PCR analysis

Genomic DNA (gDNA) was extracted using GenElute™ Plant Genomic DNA Miniprep Kit (Sigma-Aldrich, USA, Cat# G2N350-1KT) from approximately 100 mg of plant tissue. Polymerase chain reaction (PCR) was used to detect the transgene in each putative transgenic plant, carried out in 50 µL reactions containing GoTaq® Green Master Mix (25 units, Promega, USA, Cat# M7122), gDNA template (200 ng), and *1-SST* specific primers (800 nM, Supplemental Table S1) in nuclease-free water. The PCR reaction mix was placed in a thermocycler (Eppendorf, USA) under the following conditions: 2 min of denaturation at 94 °C followed by 40 cycles of amplification at 94 °C for 30 s, 58 °C for 45 s, 72 °C for 15 s, and a final elongation step at 72 °C for 10 min. PCR products were separated by electrophoresis on 1% (w/v) agarose gels.