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High levan accumulation in transgenic tobacco plants expressing the *Gluconacetobacter diazotrophicus* levansucrase gene

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ABSTRACT

Bacterial levansucrase (EC 2.4.1.10) converts sucrose into non-linear levan consisting of long β(2,6)-linked fructosyl chains with β(2,1) branches. Bacterial levan has wide food and non-food applications, but its production in industrial reactors is costly and low yielding. Here, we report the constitutive expression of *Gluconacetobacter diazotrophicus* levansucrase (LsdA) fused to the vacuolar targeting pre-pro-peptide of onion sucrose:sucrose 1-fructosyltransferase (1-SST) in tobacco, a crop that does not naturally produce fructans. In the transgenic plants, levan with degree of polymerization above 10^4 fructosyl units was detected in leaves, stem, root, and flowers, but not in seeds. High levan accumulation in leaves led to gradual phenotypic alterations that increased with plant age through the flowering stage. In the transgenic lines, the fructan content in mature leaves varied from 10 to 70% of total dry weight. No oligofructans were stored in the plant organs, although the *in vitro* reaction of transgenic LsdA with sucrose yielded β(2,1)-linked FOS and levan. Transgenic lines with levan representing up to 30 mg g^{-1} of fresh leaf weight produced viable seeds and the polymer accumulation remained stable in the tested T1 and T2 progenies. The *lsdA*-expressing tobacco represents an alternative source of highly polymerized levan.

1. Introduction

Fructans are sucrose-derived sugars consisting of two up to more than a hundred thousand fructose units. In nature, fructan synthesis occurs in a broad range of microorganisms and a limited number of plant species as non-structural storage carbohydrates. Fructans of distinct origin can differ by their degree of polymerization (DP), the presence of branches, the type of linkage connecting the fructose units, and the position of the glucose residue (Waterhouse and Chatterton, 1993). Bacterial levan is a non-linear polyfructan (DP > 10^4) with β(2,6)-linked fructosyl chains and β(2-1) branches.

Bacteria synthesize levan by the action of a secreted levansucrase (EC 2.4.1.10) that directly convert sucrose into the polymer. In plants, fructans are synthesized from sucrose and stored in the vacuole by the concerted action of at least two fructosyltransferases, being sucrose:sucrose 1-fructosyltransferase (1-SST, EC 2.4.1.99) the first enzyme in the pathway (Vijn and Smeekens, 1999). Plant fructans are mostly of the inulin type and rarely exceed 100 fructosyl residues.

Bacterial levan, due to its higher DP and better solubility in water, is preferred over plant inulin as an emulsifier or encapsulating agent in a wide range of industrial products, including biodegradable plastics, cosmetics, glues, textile coatings, and detergents. Levan as a fructose source is an appropriate feedstock for the production of dimethylfuran for liquid fuels (Román-Leshkov et al., 2007). In the food industry, levan is more relevant as a prebiotic ingredient, but it is also a preferred substrate for the production of High Fructose Syrup (HFS) because of the very low glucose content. For medical application, levan is attractive as a blood plasma volume extender. Despite all this potential application, levan is not yet commercialized at a significant scale since its industrial production from sucrose is costly and low-yielding (Kang et al., 2009).

Several groups have reported the transfer of bacterial fructosyltransferase genes into crops that do not naturally produce fructans, like tobacco (Ebskamp et al., 1994; Pilon-Smits et al., 1995; Caimi et al., 1997; Turk et al., 1997; Park et al., 1999; Gerrits et al., 2001; Konstantinova et al., 2002), potato (van der Meer et al., 1994; Pilon-Smits et al., 1996; Caimi et al., 1997; Gerrits et al., 2001), maize (Caimi et al., 1996), sugar beet (Pilon-Smits et al., 1999), white clover (Jenkins et al., 2002) and poplar (Li et al., 2008). Relative

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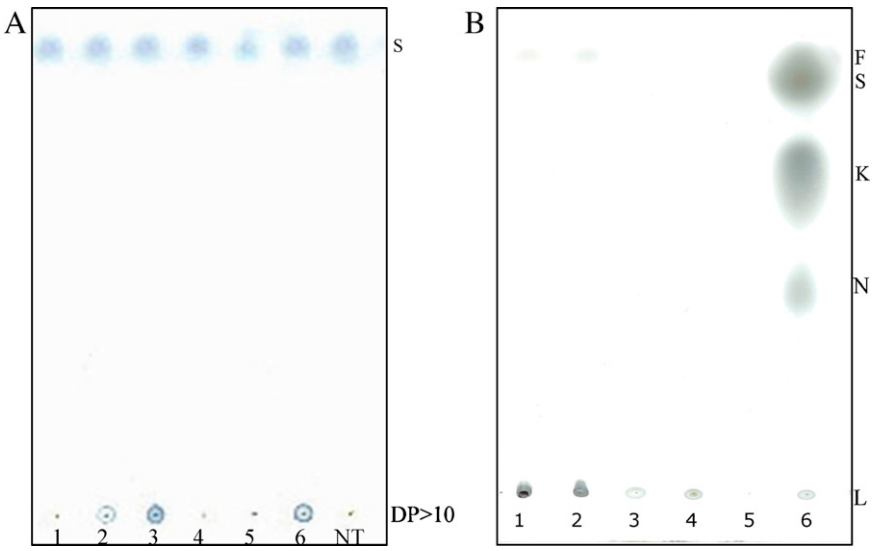

Fig. 1. TLC analysis of fructans produced in transgenic tobacco plants. (A) Fructan production in leaves of one-month old plants. Lanes 1–6: six independent transgenic lines. NT, non-transformed plant; DP, degree of polymerization. (B) Fructan accumulation in different organs of mature plants. The extracts from leaf, stem, root, flower, and seeds of three-month old plants were precipitated with 60% ethanol to concentrate the polyfructan. Shown results correspond to transgenic line 3. Lanes: 1, leaf; 2, stem; 3, root; 4, flower; 5, seeds; 6, control mixture of fructose (F), sucrose (S), 1-kestose (K), nystose (N), and levan (L).

low rates of levan accumulation (2.5–10% of dry biomass) have been achieved despite the genes were placed under the control of different constitutive or organ-specific promoters and engineered for targeting the enzyme to different subcellular plant locations: cytoplasm, apoplasm, vacuole, and plastid (Cairns, 2003). The vacuole is presumably the most compatible compartment to achieve high levan yields. In this sense, levansucrases have been fused to the vacuolar-targeting signals of yeast carboxypeptidase (CPY), sweet potato sporamin, barley lectin, potato patatin, and maize zein (Banguela and Hernández, 2006). There are no reports concerning the use of the N-terminal pre-pro-peptide of plant fructosyltransferases to drive foreign proteins to the plant vacuole.

The Gram-negative, endophytic bacterium *Gluconacetobacter diazotrophicus* secretes a constitutively expressed levansucrase (LsdA). The enzyme transfructosylation reaction on sucrose yields levan ($DP > 10^4$) and the fructooligosaccharides (FOS) 1-kestose (G1 ↔ 2F1 ← 2F) and nystose (G1 ↔ 2F1 ↔ 2F1 ← 2F) (Hernández et al., 1995). The precursor LsdA contains a 30-aa N-terminal signal peptide that is cleaved off during the secretion process (Hernández et al., 1999; Arrieta et al., 2004). Previous recombinant experiments demonstrated that yeast posttranslational modifications, such as glycosylation, do not alter the catalytic performance of LsdA (Trujillo et al., 2001, 2004).

Here, we report that the constitutive expression of LsdA fused to the pre-pro-peptide of onion 1-SST in tobacco plants led to the accumulation of high DP levan, but not oligofructans, in leaves, roots, stem, and flowers. In mature leaves of six transgenic lines, the polyfructan content varied from 10 to 70% of total dry biomass causing phenotypic alterations through the flowering stage. The *lsdA*-expressing tobacco represents an alternative source of high DP levan for food and non-food applications.

2. Materials and methods

2.1. Genetic constructs and generation of transgenic tobacco plants

The DNA encoding the N-terminal 73 aminoacids of onion sucrose:sucrose 1-fructosyltransferase (1-SST) containing the vacuolar targeting pre-pro-peptide was amplified by PCR with PfuI DNA polymerase (Promega) using the plasmid $pAcN_2$ (Vijn et al., 1998) as template and the primers 5′-gcttaagttttgttttc**a**ga**tcta**ccATGgaatc and 5′-catctcattagtc**cg**gggtaatcctc with base substitutions (bold type) to create the restriction sites BglII and SmaI, respectively. For the in-frame fusion of the pre-pro-peptide of onion 1-SST with *G. diazotrophicus* levansucrase (LsdA) lacking the first 57 residues of the precursor protein, the amplified DNA fragment was inserted into the internal SmaI site of *lsdA* in plasmid pALS5 (Arrieta et al., 1996). A six-histidine tag was genetically attached to the C-terminus of LsdA for protein purification. The chimaeric *lsdA* gene was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter, the translational enhancer omega from tobacco mosaic virus (TMVΩ), and the nopaline synthase terminator (tNos). The expression cassette was subcloned into the binary vector pCAMBIA 3300 (CAMBIA, Canberra, Australia). The resulting plasmid pHSLS126 was transferred into *Nicotiana tabacum* L. cv. SR1 via *Agrobacterium tumefaciens* LBA4404 (Hoekema et al., 1983) following the leaf disk procedure (Horsch et al., 1985). Transgenic plants were selected on MS medium (Murashige and Skoog, 1962) supplemented with 3 mg L^{-1} ammonium gluphosinate (PPT) and grown to maturity in the greenhouse. Plants were hooded to ensure self-fertilization. Seed viability was measured by germination on MS medium and soil. For fructan analysis in T1 and T2 progeny plants, seeds were germinated on PPT-containing MS medium.

2.2. Fructan extraction from plant organs and analysis by thin-layer chromatography

Samples (3 g) of plant material (root, stem, leaf, flower, or seed) were ground with liquid nitrogen in a mortar, and dissolved in 3 mL of water. The homogenate was transferred to 15 mL corning tubes, mixed by vortex, and incubated for 15 min at 90 °C. After removal of cell debris by centrifugation at 10,000 × *g* for 15 min, the supernatant (designed as plant extract) was directly assayed for total fructan content. For high-DP fructan isolation, the plant extracts were precipitated thrice in ethanol 60% at −20 °C and dissolved in deionized water. Samples (1 μL) of plant extracts or isolated levan were spotted on thin layer chromatography (TLC) silicagel foil (Merck), developed three times in acetone:water (9:1), and stained with a fructose-specific urea–phosphoric acid spray (Wise et al., 1955).