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High production of plant type levan in sugar beet transformed with timothy (*Phleum pratense*) 6-SFT genes

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ABSTRACT

Levan, a type of fructan, is an oligomer or polymer with mainly a β(2,6)-linked fructose chain attached to sucrose. We introduced two timothy genes, *PpFT1* and *PpFT2*, coding for two homologous sucrose:fructan 6-fructosyltransferases into sugar beet. Sugar beet produces a high concentration of sucrose, a starting substrate in fructan synthesis, in the root. Among transgenic T_1 lines, we obtained sugar beet transformants that accumulated large amounts of β(2,6)-linked levans (about 20 to 75 mg g^{-1} FW) in the roots. The transformed sugar beet plants possessing *PpFT1* or *PpFT2* produced linear levans with different degrees of polymerization (DP). Namely, the *PpFT1* transformants accumulated mainly high DP levans including those with DP > 40, while the *PpFT2* transformants accumulated levans with DP between 3 and 40. Chromatograms showed that PpFT2 produces pure β(2,6)-linked linear levans compared with fructans synthesized by PpFT1. These levans belong to the high DP class of plant fructans, but have much shorter DP than that of levans generally produced by microorganisms.

1. Introduction

Fructans, a class of fructose oligo- or polysaccharides with a sucrose unit, accumulate in many microbial and plant species and are well known as functional sugars of prebiotics (Ritsema and Smeekens, 2003; Roberfroid, 2005; Kang et al., 2009; Velázquez-Hernández et al., 2009; Allosopp et al., 2013). In plants, they serve as important storage carbohydrate and are thought to play a role in protecting plants against several environmental stresses (Pollock and Cairns, 1991; Ritsema and Smeekens, 2003; Van den Ende, 2013). Plant fructans are synthesized in vacuoles from sucrose by the combined action of two or more distinct fructosyltransferases, including sucrose:sucrose 1-fructosyltransferase (1-SST), sucrose:fructan 6-fructosyltransferase (6-SFT), fructan:fructan 1-fructosyltransferase (1-FFT) and fructan:fructan 6^G-fructosyltransferase (6G-FFT) (Ritsema and Smeekens, 2003). Depending on the enzyme, plant species produce different types of fructans: such as inulin and inulin neoseries (Ueno et al., 2005), levan (Cairns et al., 1999) and graminan (Yoshida et al., 2007). Recent studies have revealed more complex branched-type fructans in plants such as agave (Praznik et al., 2013) and perennial ryegrass (Lasseur et al., 2011).

Inulin, one of the major fructans found in plants, which is a linearly β(2,1)-linked fructose chain attached to sucrose, has been widely used as a functional sugar in commercial products and biomass resources (Rastall, 2010; Chi et al., 2011). The value of levan, another major fructan, has also been noted as not only a health-functional sugar but also an agent in industrial products of multiple categories because of its high water solubility, water holding ability and viscosity (Kang et al., 2009). Generally, levan is known as one of the polysaccharides produced by microbes. Microbial levans composed of a β(2,6)-linked fructose chain and β(2,1) branches are produced from sucrose by the enzymes levansucrase and fructosyltransferase in yeasts, fungi and bacteria, and many genes have been cloned from microorganisms (Velázquez-Hernández et al., 2009). In plants, temperate grasses such as timothy (*Phleum pratense*), orchardgrass (*Dactylis glomerata*), and big bluegrass (*Poa secunda*) are known to accumulate levans composed of linearly β(2,6)-linked fructose chains, and the accumulation of such levans is thought to be related to their freezing tolerance (Chatterton et al., 1993; Cairns et al., 1999; Wei et al., 2002). Among these grasses, timothy accumulates high degree of

Abbreviations: DP, degree of polymerization; HPAEC, high performance anion exchange chromatograph; 6-SFT, sucrose:fructan 6-fructosyltransferase.

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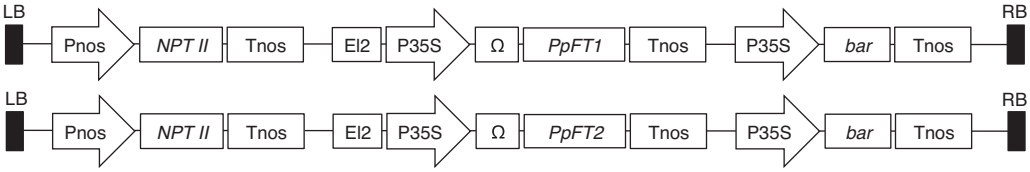

Fig. 1. Construction of a vector for agrobacterium-mediated transformation to introduce timothy 6-SFT genes, *PpFT1* and *PpFT2*, in to sugar beet. P35S, CaMV 35S promoter core (−90 to −1); El2, two-times repeats of enhancer in the 5′-upstream region of the CaMV 35S promoter; Ω, TMV 5′-upstream region; T35S, CaMV 35S terminator (Mitsuhara et al., 1996); NPTII, neomycin phosphotransferase gene; Pnos and Tnos, promoter and terminator of neomycin phosphotransferase gene in Ti plasmid, respectively; *PpFT1* and *PpFT2*, coding region of timothy sucrose:fructan 6-fructosyltransferase; *bar*, bialaphos resistance gene.

polymerization (DP) levans in its vegetative tissues. Generally, the DP of plant levans ($<10^1$ to 10^2) is about one order of magnitude shorter than that produced by microorganisms ($\times 10^2$ to $>10^3$) (Suzuki, 1993). Plant levans may have some functions for biotechnical applications that are different from those of microbial levans, but chemical and physical properties (Valluru and Van den Ende, 2008) of plant levans for industrial applications have not been studied in detail. The development of a new technique to obtain a large amount of purified plant type of levan would be useful for investigation of the characteristics of plant levans.

Metabolic engineering of fructans biosynthesis in plants has been demonstrated using genes derived from microorganisms and plants (see review: Cairns, 2003; Ritsema and Smeekens, 2003; Van Arkel et al., 2013). One of the focuses has been a potential strategy for increasing tolerance to environmental stresses such as drought and low temperatures. For example, tobacco and sugar beet plants expressing bacterial levansucrase exhibited increased drought tolerance (Pilon-Smits et al., 1995, 1999), osmotic tolerance (Park et al., 1999) and freezing tolerance (Konstantinova et al., 2002). In many successful studies using plant fructosyltransferase genes (see review: Van Arkel et al., 2013), results of transgenic studies using plant 6-SFT with an activity that extends the β(2,6)-linked fructose chain have also been reported. For instance, *Hordeum vulgare 6-SFT* was introduced into tobacco (Sprenger et al., 1995; Schellenbaum et al., 1999), and *Triticum aestivum 6-SFT* was introduced into perennial ryegrass (Hisano et al., 2004), rice (Kawakami et al., 2008), triticale (Diedhiou et al., 2012), bahiagrass (Muguerza et al., 2013) and *Brachypodium* (Tamura et al., 2014a).

Another important goal of the engineering of plant fructan metabolism is the development of genetically modified crops for high fructan production or increase value of industrial sugar as functional foods. Sugar beet is considered one of the best candidate as host plant because of its ability to accumulate sucrose in the roots (Gurel et al., 2008). A number of studies describe the successful engineering of fructan synthesis in sugar beet using *Helianthus tuberousus 1-SST* (Sévenier et al., 1998), *Bacillus subtillis sacB* (Pilon-Smits et al., 1999), and *Allium cepa 1-SST* and 6^G*-FFT* (Weyens et al., 2004). Recently, 6-SFT genes encoding enzymes that synthesize linear levan have been cloned from timothy (Tamura et al., 2009, 2014b). As a new approach to levan production in plants, we have introduced these genes into sugar beet. This is the first report of a high production level of plant type levans in transgenic plants.

2. Materials and methods

2.1. Transformation and screening of transgenic plants

The full-length sequence of the open reading frame of two timothy 6-SFT, *PpFT1* (accession no. AB436697; Tamura et al., 2009) and *PpFT2* (accession no. AB822634; Tamura et al., 2014b), was inserted into pBE2113, a Ti-based binary vector, downstream of the 5′-untranslated sequence of tobacco mosaic virus under control of the cauliflower mosaic virus (CaMV) 35S promoter (Mitsuhara et al., 1996). A CaMV 35S promoter-driven *bar* gene was also included in the vector (Fig. 1). The resulting plasmids were introduced into calli derived from plantlet leaves of the sugar beet line NK-219mm-O by *Agrobacterium*-mediated transformation according to the previously reported method (Matsuhira et al., 2012). Presence of the transgene in T_0 generation plants was examined by PCR amplification. Primer pairs for detection of *PpFT1* and *PpFT2* were 5′-GAGGTACTGCCGTGCGTGCG-3′ (forward) and 5′-TCCATCATGCCACTCCTGTC-3′ (reverse), and 5′-TTGCTTCGTCCTCATGGCGG-3′ (forward) and 5′-TTTGGTGGCGTTGGTAACTC-3′ (reverse), respectively. The screened T_0 plants were grown in soil at 23 °C (light/dark, 16 h/8 h) for 2 months, vernalized at 5 °C (light/dark, 12 h/12 h) for 6 months, and then transferred to continuous light conditions at 23 °C. After 2.5 months, mature seeds in the booting shoots were harvested. T_1 plants (grown at 23 °C, 16 h light/8 h dark) in which a transgene was detected by PCR were used for further analysis.

2.2. Carbohydrate extraction and analysis

The T_1 seedlings of sugar beet grown at 23 °C under a 16 h light/8 h dark condition for about 1 month were transferred to a greenhouse and grown at 25 °C for 6 months (from late August to late February) with additional lighting (18 h). Mature leaf blades, petioles and roots were harvested and stored at −80 °C. Total water-soluble carbohydrates were extracted from finely chopped tissues in boiling deionized water for 1 h. For analysis of fructan oligo- and polymers with different glycosidic linkages, high-performance anion exchange chromatography (HPAEC) was performed on a DX 500 chromatograph (Dionex, Sunnyvale, CA) with a Carbo Pac PA-1 anion exchange column and a pulsed amperometric detector (PAD) as described by Shiomi (1993). Peaks for glucose, fructose, sucrose, 1-kestose, 1,1-kestotetraose (Wako, Osaka, Japan) and 6-kestose (Iizuka et al., 1993) were identified by comparison with authentic standards. Linearly β(2,6)-linked fructosyl oligomers based on 6-kestose were putatively identified by comparison of HPEAC retention times with fructan oligomers extracted from wheat tissues 8 days after anthesis (Yoshida et al., 2007) and fructans from crown tissues of timothy in early winter (Tamura et al., 2014b). Glucose, fructose, sucrose, oligo- and polysaccharide concentrations of the upper, middle and bottom parts of the harvested roots were determined by HPLC with a combination of Sugar KS-802 and KS-803 columns (Shodex, Tokyo, Japan) using a refractive index detector as described by Yoshida et al. (1998).

3. Results and discussion

3.1. Production of transgenic sugar beet plants expressing PpFT1 or PpFT2

In our study, efficient embryogenic calli were obtained from leaves and petioles of NK-219mm-O plants. After *Agrobacterium*-mediated transformation, most of the regenerated plants (T_0) carrying either the *PpFT1* or *PpFT2* gene successfully grew to produce T_1 seeds under artificial conditions. Germination rates of T_1