Inulin chain length modification using a transgenic approach opening new perspectives for chicory

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Abstract

Chicory capable of synthesizing long-chain inulin is of great interest. During the growing season, the sucrose–sucrose 1-fructosyltransferase (1-SST) activity is vital for production of long-chain inulin in chicory. With the purpose to increase inulin chain length, we employed *Agrobacterium*-mediated transformation method. Transgenic chicory plants (*Cichorium intybus* L. var. *sativum*) cv. ‘Melci’ has been developed to overexpress sucrose–sucrose 1-fructosyltransferase (1-SST) under the control of the CaMV 35S promoter. The integration of the T-DNA into the plant genome was confirmed by PCR on genomic DNA using gene-specific primers. Quantification of the 1-SST transcript expression level revealed that transgenic plants showed higher 1-SST expression than those in non-transgenic plants. Further analyses proved that the fructan content of the roots significantly increased in the transgenic plants. These results revealed that overexpression of the *1-SST*, the key gene in inulin biosynthesis in chicory, might serve as a novel approach to develop plants with the long-chain inulin content.

Keywords Inulin · Chicory · Overexpress · Transgene

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Introduction

Fructans are naturally occurring plant oligosaccharides and polysaccharides in which one or more fructosyl-fructose linkages consist mostly of glycosidic bonds. Structurally, fructans can be linear or branched polymers of fructose (Kelly 2008). Fructans are present in 15% of the flowering plant species, mainly in Asteraceae, Campanulaceae, Boraginaceae (dicotyledonous plants), Poaceae, and Liliaceae (monocotyledonous plants) (Hendry 1993; Ritsema and Smeekens 2003) and can also be synthesized by green algae (Chlorophyta), fungi, and bacteria (Hendry 1993). The glycosidic bond type is the main element for the fructan classification. In linear fructan molecules, termed inulins, fructosyl (fructofuranosyl) units are connected by $\beta(2-1)$ linkages. This specific type of glycosidic bond provides inulin unique structural and physiological properties. Inulin is used as an energy reserve by some plants (Van den Ende et al. 2011), and also plays different roles in the regulation of osmotic pressure, sink strength, and resistance to cold and drought (Valluru and Van den Ende 2008; Livingston et al. 2009). Furthermore, inulin is currently added to various food products, such as yoghurt and bread (Kip et al. 2006; Morris and Morris 2012). Long-chain fructans, such as inulin, function as emulsifiers and give a better taste feeling to fat-free

yoghurt. The $\beta(2-1)$ glycosidic linkages in inulin results in a non-digestible carbohydrate; therefore, it is used as a soluble dietary fibre. Hence, inulin can pass from the small into the large intestine without being digested (Roberfroid 2007a). Consequently, in the bowel, it is utilized preferably by beneficial bacteria, such as Bifidobacteria and Lactococcus, and as a result, it will have an impact on the variation in the intestinal microbiota. This condition increases gut health (Gibson et al. 1995; Roberfroid 2007b) and might be advantageous for the immune system and inflammatory processes and conditions (van Arkel 2013). Nowadays, the interest in fructans has increased due to the health-promoting effects of fructans for humans (Altenbach 2005). Actually, inulin-type fructan is used in food and non-food products, depending on the degree of polymerization (DP). Short-chain inulin can be utilized to prepare fructose syrup as sweetening agent in drink products, whereas long-chain inulin (with a DP > 25) is applied in food products as a prebiotic or fat replacer (Franck and De Leenheer 2002). In addition, long-chain inulin can be used in detergent products (van Arkel 2013).

Although inulin typically occurs in the order of Asterales, mostly tuberous plants, such as *Helianthus tuberosus* (Jerusalem artichoke), *Cichorium intybus* (chicory), *Dahlia pinnata* (dahlia), and *Polymnia sonchifolia* (yacon) (de Oliveira et al. 2011), most of the inulin production on an industrial scale is currently derived from chicory roots due to its high content (van Arkel et al. 2012). The most important factor affecting the inulin quality is its polymer length (DP). In chicory, the polymer length of extracted inulin at harvest time has been reported to decrease (van Arkel 2013) as this is a highly unfavourable feature for high DP inulin production.

The reduced polymer length of inulin is affected by the catalytic reactions of two enzymes: fructan:fructan 1-fructosyltransferase (1-FFT) and fructan 1-exohydrolase (1-FEH). Expression levels and activities of these enzymes depend on the genetic background of the chicory cultivars (Koch et al. 1999) and on environmental factors (van Arkel 2013). 1-FEH is the main reason for the catalytic breakdown of inulin and a number of efforts have been proposed to deal with this problem. The most important approach is down-regulation of 1-FEH to block its activity (Maroufi 2006). In addition, during the growing season, the sucrose–sucrose 1-fructosyltransferase (1-SST) activity is vital for the availability of 1-kestose (the shortest fructan) and long-chain inulin. It is clear that under 1-kestose-limiting conditions, which occur during the low 1-SST activity, the mean DP of inulin decreases (van den Ende et al. 1996a; van Arkel et al. 2012). Moreover, the 1-SST activity has been suggested to rapidly increase in the first 7–8 weeks of chicory growth and then decrease until harvest time (van den Ende et al. 1996b; van Arkel et al. 2012). Therefore, the decreased 1-SST activity could adversely affect the production of long-chain inulin

in chicory roots. Accordingly, up-regulation of 1-SST is required for long-chain inulin formation. With regard to this issue, first, transgenic chicory plants enable to overexpress the 1-SST gene were generated. Then, the ability to produce long-chain inulin of transgenic plants overexpressing the 1-SST gene was investigated.

Materials and methods

Plant material

Seeds of a root-type chicory cultivar “Melci” were obtained from the Institute for Agricultural and Fisheries Research (ILVO, Belgium). The seeds were surface sterilized by soaking in 70% (v/v) ethanol for 1 min followed by treatment with 1% (v/v) sodium hypochloride for 10 min and washed at least three times with sterilized water. Seeds were sown in glass jars on half-strength MS medium (Murashige and Skoog 1962) containing 0.8% (w/v) agar. The incubation conditions for germination were 26 ± 1 °C under a 16-h photoperiod of 1000–1200 lx illumination intensity. The first leaves were excised from the 3-week-old seedlings to prepare explants for the *Agrobacterium tumefaciens*-mediated transformation.

Plasmid construction and genetic transformation

A 1899-bp double-strand cDNA fragment containing the complete 1-SST-encoding region (accession no. AJ250634) of *Taraxacum officinale* (dandelion) was amplified by a proofreading DNA polymerase (*Pfu* DNA Polymerase, Promega, Fitchburg, WI, USA) by means of a specific primer pair fused forward and reverse to the attB1 and attB2 sequences, respectively (Table 1). The PCR product including the target sequences was recombined into the pDONOR221 with BP clonase II (Invitrogen, Carlsbad, CA, USA). As confirmation for the amplified DNA, a few size-verified pENTRY plasmids were sequenced. With the BP Clonase II recombination kit (Invitrogen), the full-length 1-SST DNA (integrated into the pENTRY clones) was recombined into the pK2GW7 (Karimi et al. 2002). The pK2GW7 destination plasmid is a binary vector for plant transformation containing a cauliflower mosaic virus (CaMV) 35S promoter and an NOS polyadenylation terminator, designed for gene overexpression studies. The generated expression vector was designated pK2GW7-1-SST (Fig. 1). This expression vector containing the 1-SST-coding sequence under the control of the CaMV35S promoter was finally transferred into the *Agrobacterium* C58C1 RifR (pMP90) (Koncz and Schell 1986) by means of the freeze–thaw method (An et al. 1988). *Agrobacterium* harbouring the expected expression vector [C58C1 (pMP90)

Table 1 Primer sequences used for gene amplification and for real-time qPCR analysis

Name	Forward	Reverse
<i>1-SST1</i>	ATGGCTTCCTCAACCACCGC	TTAAGAACTCCACCCAGAAAGTG
<i>ACTIN</i>	GAACAAGAGCTCGAAACCGC	AATCTTTCGGCTCCGATGGT
<i>1-SST2</i>	AGGAGGAAGAACGGTGGTGA	TTGACGCTAATTCCGGTGGC
<i>1-FFT</i>	TTGAGGTGGAACAAGAGACG	GGTCCCAAACCTTCCCCTTTC
<i>attB</i>	<i>attB1</i> 5'-GGGGACAAGTTTGTACAAAA AGCAGGCTTC	<i>attB2</i> 5'-GGGGCAGCTTTCTGTACAAAGTGGGA

Fig. 1 Diagram of *1-SST* overexpression constructs in the pK2GW7 binary vector

(pK2GW7-1-SST)] was selected for chicory transformation. The *Agrobacterium* culture and subsequent transformation were carried out basically as described (Maroufi et al. 2012).

PCR analysis of transformants

All primer sets were designed and analysed with the Vector NTI primer design software (Thermo Fischer Scientific, Waltham, MA, USA) (Table 1). Genomic DNA from leaf tissue was isolated with the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987). The integration of the expression cassette (T-DNA) into the plant genome was verified by PCR on genomic DNA using a primer pair directed against a CaMV 35S promoter region (Table 1). Genomic DNA (100 ng) from each putative transgenic (T_0 generation) and non-transgenic (control) plants was used as a template for PCR amplification and the DNA of the linearized pK2GW7-1-SST expression vector as positive

control. The PCR products were analysed by electrophoresis on a 1.2% (w/v) agarose gel.

Semi-quantitative RT-PCR and quantitative PCR

Total RNA was extracted from the well-developed tap-roots of transgenic and non-transgenic (control) chicory plants grown in the same environmental condition, with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the supplier's protocol. RNAs were treated with Ambion TURBO™ DNase (Thermo Fischer Scientific) to eliminate DNA contamination. cDNAs were synthesized from 1 µg total RNA for each sample with the cDNA SuperScript® VILO™ synthesis kit (Thermo Fischer Scientific) according to the manufacturer's instructions.

To confirm the expression of the integrated *1-SST* transgene in the transgenic chicory lines, semi-quantitative RT-PCR with specific primers for *1-SST* and actin genes

(1-SST2 and actin; Table 1) were carried out. In addition, a real-time quantitative PCR (qPCR) was performed in a 10- μ l reaction volume, with 2.5 μ l of tenfold diluted cDNA as a template and 5 ml of 1 $\times$ Power LightCycler[®] 480 SYBR[®] Green I Master (Roche Diagnostics, Vilvoorde, Belgium) and 0.3 μ mol of forward and reverse primers. No-template controls were also included for all genes. The PCR conditions were as follows: activation and denaturation at 95 °C for 5 min, followed by 45 cycles of amplification at 95 °C for 10 s followed by 60 °C for 10 s, and a final extension at 72 °C for 10 s. Dissociation curves were generated by increasing the temperature starting from 65 to 95 °C to determine the specificity of the PCR amplification reactions. For each reaction, the crossing cycle number (C_p) created by the LightCycler480 SW 1.5 software (Roche Diagnostics) with default parameters with the second derivative method was collected. For each primer pair of the targets (*1-SST* or *1-FFT*) and reference (actin) genes, the PCR amplification efficiencies were calculated from a dilution series of the cDNA of transgenic plants with the equation $E = [10^{(-1/\text{slope})}]$ (Pfaffl 2001). The differences between the C_p values of the reference and the target gene were normalized with the formula $2^{-\Delta\Delta C_i}$ (Pfaffl 2001). Finally, the relative transcript levels (fold-differences) of the target genes between primary transgenic transformants and non-transgenic plants were calculated.

Inulin chain length analysis

Well-developed tap roots were harvested from five primary transformants and five control plants which were grown in the same environmental condition, cleaned, and sliced. The large pieces of roots were shredded with a cheese grater to obtain small pieces of maximum 2 mm thickness and finally well mixed (Van Waes et al. 1998). The subsamples were immediately frozen and stored at – 20 °C for sugar measurement. The total fructose (Fru) and total glucose (Glu) contents were quantified by anion exchange high-performance liquid chromatography (HPLC) (Baert 1997; Van Waes et al. 1998). The total sugar content was described as the sum of the total Fru and Glu ([Fru + Glu]/1.1) (Baert 1997; Van Waes 1998). The ratio of total Fru to total Glu corresponded well with the average chain length or DP of inulin ([Fru/Glu]/1) (Baert 1997; Van Waes et al. 1998).

Results

Agrobacterium-mediated plant transformation

To generate stable transgenic chicory plants overexpressing the 1-SST-encoding gene, *Agrobacterium*-mediated transformation experiments were carried out. After co-cultivation of leaf piece explants with *Agrobacterium* and regeneration

on 500 mg/l kanamycin-containing MS medium, a number of shoots were successfully obtained. No morphologically abnormal plants were observed within the putative transgenic plantlets. The kanamycin-resistant shoots with a few strong roots were transplanted into sterilized soil and successfully acclimatized in the greenhouse.

Using a specific primer pair targeting the CaMV 35S promoter (Table 1), the integration of T-DNA was analysed by PCR. Amplified products of the expected size of 500 bp were detected on ethidium bromide-stained agarose gels (Fig. 2a). Moreover, the presence of transgenic 1-SST transcripts was examined by semi-quantitative RT-PCR with the 1-SST2 and actin primers (Table 1; Fig. 2b). The actin transcripts were equally amplified from each putative transgenic lines (T1, T2 ...) and control plants. However, obvious differences between the 1-SST transcript of the control and transgenic plants were observed (Fig. 2b), suggesting that the transgenes were integrated into the transgenic lines.

Gene expression analysis of transgenic lines

Transcript quantification of the two key genes (*1-SST* and *1-FFT*) involved in inulin biosynthesis was determined by RT-qPCR. The expression levels of these genes were evaluated in the tap root tissues of five transgenic primary transformants (T) and five control (WT) plants. Since in the previous study on chicory, the stability of actin was validated (Maroufi et al. 2010), it, therefore, was used as the reference gene in the present work.

Fig. 2 **a** PCR amplification of the CaMV 35S promoter from putative transformed and nontransformed plants. Lane M, the 1-kb DNA ladder; lane 1, DNA sample from vector pK2GW7-1-SST; lane 2, water; lanes 3–13, DNA samples from putative transformed plants. **b** RT-PCR amplification of 1-SST and actin from putative transformed and nontransformed plants (WT)

The relative quantification was calculated with the following formula by utilizing the stably expressed internal control gene, actin (as reference gene):

Relative Expression

$$= \frac{(\text{Efficiency target})^{\Delta\text{CP target (control-sample)}}}{(\text{Efficiency reference})^{\Delta\text{CP reference (control-sample)}}}$$

For each gene, specificity of amplification was demonstrated by a single peak in the melting curve analysis (Fig. supplementary 1). In general, the expression level of *1-SST* was lower in the control plants in comparison with the transgenic plants (up to four fold) (Fig. 3). The highest level of *1-SST* transcript was obviously observed in the transgenic plants (Fig. 3). However, no significant differences were detected for *1-FFT* transcript between the wild-type controls and the transgenic lines (not overexpressed in these lines). *1-FFT* which is also involved in inulin biosynthesis was selected to confirm the appropriate gene expression analysis in the transgenic lines. The investigation of *1-FFT* expression pattern revealed that the transcript between control and transgenic plants did not show significant difference (Fig. 3). Therefore, these results revealed that transcript levels of *1-SST* in the transgenic chicory plants were significantly increased, indicating that the *1-SST* transgene is integrated into the genome, whereas the variation in the transcriptional *1-SST* levels could be due to the T-DNA number or T-DNA positional effects in transgenic chicory plants.

Inulin chain length analysis

As the *1-SST* is expressed at higher level in the roots of the primary transgenic T3, T4, and T5 lines (Fig. 4), the impact of *1-SST* on the inulin level was needed to be examined. Hence, Fru, Glu, the total sugar content, and average

DP of inulin in transgenic and WT chicory root tissue were quantified. All the data obtained by the HPLC experiments (Table 2) showed that the transgenic plants contained more total sugar contents (Fru + Glu) than the control plants (Fig. 5). In addition, compared with the controls, different transgenic lines (T1–T5) showed a significantly higher average inulin chain length (DP) (Fig. 4). This observation is consistent with the differential expression results and shows that *1-SST* overexpression influences inulin in chicory roots.

Discussion

Inulin is an important carbohydrate storage compound mainly produced by plants. Several studies have been performed to identify inulin biosynthesis pathway. To date, the genes encoding various fructosyltransferases have also been isolated from several fructan-containing plants and microorganisms, such as bacteria and fungi, and their

Fig. 4 Expression level of *1-SST* relevant to the DP in different transgenic and control plants

Fig. 3 Relative expression level of *1-SST* and *1-FFT* in different transgenic and control plants

Fig. 5 Mean fructose, glucose, total sugar content, and average DP in transgenic and control plants

Table 2 Composition of chicory root fructose, glucose, total sugar and DP, analysed by HPLC

Sample	Glu	Fru	Total sugar	DP = [Fru/Glu]/1
WT1	2.47	21.2	21.41	9.91
WT2	2.52	19.31	19.74	8.92
WT3	2.57	19.28	19.75	8.76
WT4	2.26	19.44	19.61	9.96
WT5	2.46	20.82	21.05	9.79
T1	1.8	20	19.82	11.11
T2	2.06	25	24.60	12.14
T3	1.77	23.19	22.69	13.10
T4	2.24	23.9	23.76	10.67
T5	2.22	23.49	23.37	10.58
Mean WT	2.46	20.01	20.31	9.47
Mean transgenic	2.02	23.12	22.85	11.52
SD WT	0.12	0.92	0.85	0.58

functionalities have been assessed (Hisano et al. 2004). As chicory is the main source of inulin, its biosynthesis and regulation has been comprehensively studied (Van Laere and Van den Ende 2002). Inulin is biosynthesized by two distinct enzymes: sucrose:sucrose 1-fructosyltransferase (1-SST) and fructan:fructan 1-fructosyltransferase (1-FFT) (Edelman and Jefford 1968). The 1-SST enzyme acts in the first biosynthesis step and initially transfers fructosyl between two sucrose molecules. Further inulin chain elongation is catalysed by 1-FFT, a fructosyltransferase that adds β (2, 1)-linked fructofuranosyl units (Van den Ende and Van Laere 1996). Moreover, fructan exohydrolases (FEHs),

including 1-FEH, FEHIIa, and FEHIIb, have been reported to contribute in the degradation of inulin (Van den Ende et al. 2000, 2001). Therefore, to produce long-chain inulin in chicory, the genes coding for 1-SST, 1-FFT, and FEHs are the main target genes for genetic modification.

During chicory root growth, *1-SST* starts with its maximum expression in June and then decreases in a linear manner from July until December (van Arkel et al. 2012), but for long-chain inulin, the constant expression level of *1-SST* is essential. Hence, one central way to produce inulin with long-chain length is to increase the expression level of *1-SST* (van Arkel 2013). Several studies have been done to increase the *1-SST* and *1-FFT* expression levels; for instance, light has been found to induce the *1-SST* expression and high enzyme activity correlates with the fructan concentration (Ameziane et al. 1994). Moreover, the expression of *1-SST* and *1-FFT* in hairy root cultures of chicory in relation with nitrogen accessibility in media with high-carbon and low-nitrogen content was highly induced and, consequently, more inulin accumulated (Kusch et al. 2009). Nevertheless, these techniques are seemingly not very practical to increase the gene expression and finally improve the inulin content. Thus, the best choice is possibly *1-SST* overexpression.

In this study, we succeeded in obtaining transgenic chicory plants that carry the *1-SST* transgene from dandelion. We used a well-known promoter (CaMV 35S) in plant transformation. It causes constant and high level of gene expression in dicot plants (Benfey and Chua 1990). The result showed that the expression level of *1-SST* in roots of transgenic plants was higher compared with those in wild-type plants. It seems that the expression was not conditioned by endogenous factors, since a constitutive promoter CaMV 35S was used. Nevertheless, in the field or under constant photoperiod and temperature, the expression patterns of innate 1-SST at different phases of the growing season are completely different and vary in the tap root of chicory plants (van Arkel et al. 2012). Accumulation of transgenic mRNA (1-SST) was detected in the transgenic chicory plants at harvest time, whereas in normal plants (van Arkel 2013), the expression of *1-SST* decreases until the end of the growing season in November, when only 10% of the activity remains. Further investigation of the fructose analysis revealed that the accumulation and DP of inulin content is higher in roots of transgenic chicory plants in comparison with WT plants. This observation is almost positively related to the constant expression level of *1-SST* in transgenic chicory plants. Stable *1-SST* expression may provide 1-kestose, the shortest member of the inulin series as a fructosyl donor, which is required for 1-FFT to increase the mean DP (Van den Ende 1996b; van Arkel et al. 2012). However, when the 1-SST activity is low, 1-kestose is limited and 1-FFT additionally can catalyse the transfer of fructosyl units from an

inulin molecule onto sucrose, with a decreased mean DP as a result (Van den Ende et al. 1996b; van Arkel et al. 2012).

Ye et al. (2001) reported that overexpression of *SacB* coding for levansucrase (sucrose 6-fructosyl transferase) isolated from *Bacillus subtilis*, triggered fructan accumulation in Italian ryegrass (*Lolium multiflorum*). In addition, the gene-encoding fructosyltransferase (6-*SFT*), a key enzyme of fructan synthesis from barley (*Hordeum vulgare*), has been overexpressed in tobacco (*Nicotiana tabacum*) and chicory (Sprenger et al. 1997) to alter fructan biosynthesis. *I-SST* from *Helianthus tuberosus* has been introduced into sugar beet (*Beta vulgaris*) (Sévenier et al. 1998), and in the taproot of transgenic plants, the stored sucrose was almost totally converted into low molecular weight fructans. *Petunia* (*Petunia hybrida*) plants also transformed with the *I-SST* from *Helianthus tuberosus* accumulated the oligofructans 1-kestose (GF2), 1,1-nystose (GF3), and 1,1,1-fructosyl-nystose (GF4) (van der Meer et al. 1998). Similarly, *I-SST* from globe artichoke (*Cynara cardunculus*) has been transformed into potato (*Solanum tuberosum*) to study potential fructan-produced changes (Hellwege et al. 1997).

Altogether, higher *I-SST* expression levels in transgenic chicory plants show a significant role in the accumulation of long-chain inulin in chicory roots. However, further analyses, particularly on the role of FEH, await to elucidate the production of root chicory with high inulin DP.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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