Development of rubber-enriched dandelion varieties by metabolic engineering of the inulin pathway

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Summary
Natural rubber (NR) is an important raw material for a large number of industrial products. The primary source of NR is the rubber tree Hevea brasiliensis, but increased worldwide demand means that alternative sustainable sources are urgently required. The Russian dandelion (Taraxacum kokssaghyz Rodin) is such an alternative because large amounts of NR are produced in its root system. However, rubber biosynthesis must be improved to develop T. koksaghyz into a commercially feasible crop. In addition to NR, T. koksaghyz also produces large amounts of the reserve carbohydrate inulin, which is stored in parenchymal root cell vacuoles near the phloem, adjacent to apoplastically separated laticifers. In contrast to NR, which accumulates throughout the year even during dormancy, inulin is synthesized during the summer and is degraded from the autumn onwards when root tissues undergo a sink-to-source transition. We carried out a comprehensive analysis of inulin and NR metabolism in T. koksaghyz and its close relative T. brevicorniculatum and functionally characterized the key enzyme fructan 1-exohydrolase (1-FEH), which catalyses the degradation of inulin to fructose and sucrose. The constitutive overexpression of Tk1-FEH almost doubled the rubber content in the roots of two dandelion species without any trade-offs in terms of plant fitness. To our knowledge, this is the first study showing that energy supplied by the reserve carbohydrate inulin can be used to promote the synthesis of NR in dandelions, providing a basis for the breeding of rubber-enriched varieties for industrial rubber production.

Introduction
Natural rubber is a unique and economically important biopolymer mainly produced by the rubber tree Hevea brasiliensis (Schulze Gronover et al., 2011). However, the increasing demand for NR (>12.1 million tons in 2014) and adverse influences, such as climate change, vulnerable H. brasiliensis monocultures and their replacement by more profitable oil palms, have encouraged the search for alternative NR-producing plants (Arias et al., 2016). The annual or perennial Russian dandelion (Taraxacum kokssaghyz) synthesizes high molecular mass poly(cis-1,4-isoprene) in specialized latex-producing tubular cells known as laticifers and therefore offers an alternative source of NR (Epping et al., 2015). Although laticifers are also found in pedicels and leaves, NR is mainly synthesized in the T. kokssaghyz root system.

The basic building block of NR is isopentenyl pyrophosphate (IPP), which is thought to be produced mainly via the cytosolic mevalonic acid (MVA) pathway (van Deenen et al., 2012). The same pathway also provides IPP for the synthesis of various isoprenoid end products, including steroids and pentacyclic triterpenes (Figure 1) that fulfil important roles in membrane fluidity, development and resistance against herbivores (Huber et al., 2015; Schaller, 2003). In H. brasiliensis, sucrose is thought to be the exclusive precursor of IPP and is actively translocated into the laticifers by sucrose transporters (Dussoit-Coucaud et al., 2009, 2010).

In T. brevicorniculatum, an apomictic close relative of T. kokssaghyz (which reproduces sexually and undergoes obligatory outcrossing), NR is synthesized on the surface of rubber particles, which are stabilized by auxiliary proteins such as the rubber elongation factor (REF) (Laibach et al., 2015). The elongation of the IPP chain is catalysed by a rubber cis-prenyltransferase (CPT) complex whose activity is enhanced by the presence of a rubber transferase activator (RTA) (Epping et al., 2015). In T. brevicorniculatum, NR typically represents about 0.5% of the root dry weight (DW) (Epping et al., 2015; Post et al., 2012). In contrast, T. kokssaghyz shows considerable intraspecific genetic and phenotypic diversity, and the NR content varies between 2% and 15% DW in this species (Koroleva, 1940; Van Beilen and Poirer, 2007).

Inulin, a linear β-(2→1)-linked fructan, is another abundant dandelion metabolite that accumulates exclusively in the roots to levels exceeding 50% DW (Van den Ende et al., 2000b). Fructans are water-soluble reserve carbohydrates that are thought to be synthesized and stored within the vacuoles in ~15% of all angiosperm species, including chicory (Cichorium intybus), Jerusalem artichoke (Helianthus tuberosus), artichoke (Cynara scolymus) and dandelions, and several cereals and grasses (Carpita et al., 1991; Darwen and John, 1989; Hellwege et al., 2000; Hendry, 1987; Van den Ende et al., 2000b; Wagner et al., 1983). Dandelion inulin crystals are clustered in parenchymal root cell vacuoles close to phloem tissues adjacent to laticifers.
Figure 1  Putative model of the inulin and isoprenoid metabolic network in dandelion roots. Sucrose, either transferred from the apoplast or synthesized from glucose and fructose in the cytosol of the parenchymal root cells, is transported into the vacuole and used as a substrate for inulin biosynthesis. The degradation of inulin by 1-FEH produces free sucrose and fructose. Sucrose is actively transported through the cytoplasm and apoplast into the laticifers and is used as a precursor via the mevalonate pathway for the synthesis of isopentenyl pyrophosphate (IPP), the basic building block of isoprenoids such as triterpenes and poly(cis-1,4-isoprene). F-6-P, fructose-6-phosphate; G-1-P, glucose-1-phosphate; GFF, 1-kestose; MVA, mevalonic acid; DMAPP, dimethylallyl pyrophosphate; FPP, farnesyl pyrophosphate; IPP, isopentenyl pyrophosphate.
et al. (1996). In autumn, declining levels of photoassimilates promote inulin degradation, and root tissue thus undergoes a sink-to-source transition (De Roover et al., 1999; Van den Ende and Van Laere, 1996; Van den Ende et al., 2000b). The inulin metabolic pathway comprises three major enzymes representing glycosyl hydrolase (GH) family 32 (Henrisat, 1991; Verhaest et al., 2005) (Figure 1). The synthesis of inulin is catalysed by two fructosyltransferases (Edelman and Jefford, 1968; Lüscher et al., 1996). A sucrose: sucrose 1-fructotransferase (1-SST) (EC 2.4.1.99), which determines sink strength, produces the trisaccharide 1-kestose by transferring a fructose residue from one sucrose molecule to another. Further fructose chain elongation is catalysed by fructan: fructan 1-fructosyltransferase (1-FFT) (EC 2.4.1.100). The degradation of inulin is catalysed by fructan 1-exohydrolase (1-FEH) (EC 3.2.1.80), which hydrolyses terminal fructose residues from fructan molecules sequentially until only a sucrose unit remains (De Roover et al., 1999; Edelman and Jefford, 1968). The degradation of sucrose is then catalysed by invertases (Sturm, 1999). In addition to its function as a rapidly accessible energy reservoir, inulin may protect the plant during drought or cold stress by stabilizing membranes (De Roover et al., 2000; Livingston and Henson, 1998; Pilon-Smits et al., 1999; Valluru and Van den Ende, 2008).

In dandelion, the fact that inulin is localized adjacent to laticifers suggests that excess free sugars (e.g. fructose and sucrose) generated by inulin degradation could be used for the formation of other isoprenoids such as sterols and pentacyclic triterpenes, and the eventual saturation of this relief pathway led to the accumulation of MVA pathway precursors, affecting upstream flux and redirecting carbon to the storage product inulin (Post et al., 2012).

It is important to understand the metabolic processes that affect NR synthesis, particularly in order to enhance the productivity of annually grown T. koksaghyz as a source of NR. Here, we provide evidence that a proportion of the energy supplied by inulin degradation is redirected to the biosynthesis of NR in wild-type plants after sink-to-source transition in the roots and that the rubber content can therefore be improved by the overexpression of Tk 1-FEH to promote further inulin degradation. Our study thus provides an appropriate basis for the breeding of rubber-enriched dandelion varieties for industrial rubber production.

Results and discussion
Identification and characterization of T. brevicorniculatum and T. koksaghyz 1-SST, 1-FFT and 1-FEH
We recently identified and characterized several dandelion genes involved in the synthesis of NR (Epping et al., 2015; Laibach et al., 2015; Post et al., 2012), but little is known about genes responsible for inulin metabolism. The full-length 1-SST, 1-FFT and 1-FEH cDNAs from T. brevicorniculatum and T. koksaghyz roots were therefore isolated based on known fructosyltransferase sequences and expressed sequence tag (EST) data.

In silico translation predicted open reading frames and molecular masses of 632 amino acids (aa) and 71.5 kDa for Tb1-SST and Tk1-SST, 622 aa and 69.8 kDa for Tb1-FFT, and 622 aa and 69.7 kDa for Tk1-FFT. The low predicted isolectric points of pI 5.0 for Tb1-SST and Tk1-SST, and pI 5.2 for Tb1-FFT and Tk1-FFT, are common features among fructosyltransferases and 1-FEHs (Lüscher et al., 2000; Sprenger et al., 1995; Van den Ende et al., 2001). Sequence alignment showed that Tb1-SST and Tk1-SST shared 100% pairwise identity, whereas Tb1-FFT and Tk1-FFT shared 97.8% pairwise identity. Each sequence shared more than 99% identity with its T. officinale orthologue and more than 79% identity with the corresponding proteins from chicory (Ci1-SST AFB83319 and Ci1-FFT AAD00558) and Jerusalem artichoke (H1-SST CA0A8812 and H1-FFT CA0A8811). Multiple sequence alignments using MUSCLE revealed the presence of the three GH32 family-specific conserved regions including the three catalytically active amino acids shown in bold: x-x-x-D-P-D-N-G; RDP; and EC (Altenbach and Ritsema, 2007; Altenbach et al., 2005; Edelman and Jefford, 2008) (Figure S1). Furthermore, the fructosyltransferase-specific motif x-A/G-Y/F was found in Tb1-SST, Tk1-SST, Tb1-FFT and Tk1-FFT (Altenbach et al., 2005; Lasseur et al., 2009).

The in silico translation of the amplified Tb1-FFT and Tk1-FFT cDNAs predicted proteins containing 581 aa, with molecular masses of 65.7 kDa and pl values of 5.8. SignalP predicted the presence of a 25-residue N-terminal signal peptide. Pairwise sequence alignment showed that Tb1-FFT and Tk1-FFT shared 98.6% identity and were also closely related to the chicory enzymes Ci1-1-FEHla (CAC37922) with >90% identity and Ci1-1-FEHlb (CAC37923) with >88% identity. Both dandelion 1-FEHs showed lower levels of identity with the chicory enzymes Ci1-1-FEH (CAC19366) with 52% identity, and an invertase (CAAA72009) with 59% identity. The hydrolase-specific W-A/S/G-W motif and the three conserved regions common to GH32 enzymes, including the three highly active amino acids mentioned above, were also found in Tb1-FFT and Tk1-FFT (Altenbach et al., 2005; Lasseur et al., 2009; Le Roy et al., 2007, 2008) (Figure S1). The combined in silico data suggested that we had identified four fructosyltransferases as well as two 1-FEHs that were suitable for further investigation.

Analysis of inulin and NR metabolism in T. koksaghyz throughout the growing season
To gain an overview of inulin and NR metabolism in T. koksaghyz throughout the growing season, we analysed plants grown under near-natural conditions outside the greenhouse with supplemental irrigation.

Approximately 200 mg/g DW inulin was present in the roots in May, but this increased to ~250 mg/g DW by June and the mean degree of polymerization (DP) increased from nine fructose molecules (FMs) in May to 12 by July (Figure 2a). During this time, the amounts of fructose (minimum 17.7 mg/g DW in July) and sucrose (minimum 17.7 mg/g DW in June), both of which are used to synthesize inulin and are released during its degradation, remained at low levels (Figure 2b). By November, nearly 50% of the stored inulin detected in July had degraded. Furthermore, the DP had decreased from 12 FMs in July to 7 FMs in November. This correlated with increasing fructose and sucrose levels. The amount
of fructose increased strongly in October, reaching 24.3 mg/g DW by November, whereas sucrose levels increased to more than 35 mg/g DW in the autumn. Furthermore, throughout the growing season, the inulin level and DP precisely matched the accumulation of large amounts of high-quality inulin in the roots (Figure 2d). The poly(cis-1,4-isoprene) content in May (0 mg/g DW) increased to 50 mg/g DW by November (Figure 2a). Although the degradation begins when the root undergoes a sink-to-source transition in late summer caused by photoperiodic changes from long-day to short-day conditions (De Roover et al., 1999; Van den Ende et al., 2000b). Tk1-FEH was expressed at minimal levels during the summer months but was induced in the autumn and reached its maximum expression level in November. The down-regulation of fructosyltransferases combined with strongly induced Tk1-FEH activity contributed to the breakdown of inulin and increased the levels of fructose (to 40 mg/g DW) and sucrose (to >50 mg/g DW) by November (Figure 2c–d). Although the degradation of inulin addresses the need for a rapidly accessible energy supply, other factors may also play a significant role, such as overwintering and abiotic stress tolerance (Livingston and Henson, 1998; Tamura et al., 2014; Van den Ende and Van Laere, 1996; Van den Ende et al., 2000a).

Parenchymal cells containing inulin are located close to laticifers that produce NR, so it is possible that free sugars (e.g. fructose and sucrose) supplied by inulin degradation are used by the laticifers. The accumulation of poly(cis-1,4-isoprene) was first observed in young T. koksaghyz plants after the formation of laticifers. Eight-week-old plants contained only low levels of NR in the roots (Figure 2d). The poly(cis-1,4-isoprene) content in May was 9 mg/g DW, but this had risen to 40 mg/g DW by September. Although the lower photosynthetic rate in autumn...
provides lower amounts of substrate sugars for NR biosynthesis, the quantity of poly(cis-1,4-isoprene) nevertheless increased above 70 mg/g DW in November. The increasing level of poly (cis-1,4-isoprene) throughout the growing season was accompanied by the accumulation of fructose and sucrose in *T. koksaghyz* roots, indicating a connection between inulin degradation and the synthesis of NR. *H. brasiliensis* produces large amounts of high-quality NR within its apoplastically separated laticifers, using sucrose as the exclusive precursor (Dusotoit-Coucaud et al., 2009). Several sucrose transporters (HbSUT1A, HbSUT2A and HbSUT3) actively translocate sucrose from the apoplast into the laticifers (Dusotoit-Coucaud et al., 2009, 2010; Tang et al., 2010). Additionally, the vacuolar release of sucrose via SUC4-type transporters through the tonoplast was confirmed by the characterization of AtSUC4 in *Arabidopsis thaliana* roots (Endler et al., 2006; Sauer, 2007), whereas the vacuolar export of fructose is mediated by the fructose-specific uniporter SWEET17 located on the tonoplast (Guo et al., 2014). In *T. koksaghyz*, similar transporters may enable the transport of fructose and sucrose generated by the degradation of inulin, moving them out of the vacuole, through the apoplast and into the laticifers.

**Functional characterization of ectopic Tk1-FEH**

Having identified a *T. koksaghyz* fructan 1-exohydrolase and investigated its expression profile during the growing season, revealing a potential role in inulin degradation (Figure 2), we next studied its function by heterologous expression in the methylotrophic yeast *Pichia pastoris* strain 2. This host species does not express any fructosyltransferases, making it highly suitable for the production of recombinant 1-FEHs (Xu et al., 2015). The native signal peptide was removed and replaced with an N-terminal alpha-mating factor signal peptide from *Saccharomyces cerevisiae* to ensure the secretion of Tk1-FEH into the culture supernatant. The latter has a molecular mass of 20 kDa and is mostly removed before the recombinant protein is secreted from the yeast cell (Cereghino et al., 2002). SDS-PAGE analysis revealed a ~75-kDa band that was not present in the control samples (Figure 3a).

Figure 3 Analysis of ectopic Tk1-FEH produced in *Pichia pastoris*. All investigations were carried out using dialysed supernatants from the expression cultures (a = 20 µL, b, c and d = 5 µL). Assays (b) and (d) were carried out using 5% chicory inulin dissolved in 150 µL McIlvaine buffer pH 5.1. (a) SDS-PAGE analysis of proteins from untransformed *P. pastoris* (UT), *P. pastoris* carrying an empty pPink-UTHC vector (EV) and *P. pastoris* transformed with Tk1-FEH. (b) Inulin conversion of the *P. pastoris* cultures mentioned above. (c) Degradation of different substrates (14 mM each) by Tk1-FEH measured for 3 h at 30 °C and pH 5.1. (d) Influence of sucrose on inulin degradation by Tk1-FEH measured for 4 h at 30 °C (pH 5.1). Statistically significant differences are indicated by asterisks (**P ≤ 0.001; unpaired t-test) (n = 3; mean ± SD).
negative control was only observed in samples containing the recombinant TK1-FEH (Figure 3b). The optimal pH of the recombinant TK1-FEH was 5.1 at 30 °C using chicory inulin as the substrate (Figure S2). The relatively acidic pH optimum is typical for vacuolar enzymes as already shown for 1-FEHs from Jerusalem artichoke that are located in the vacuole closely associated with the tonoplast (Darwen and John, 1989). The substrate specificity of the recombinant TK1-FEH was determined by testing inulin, nystose, 1-kestose and sucrose as substrates. Whereas 27% of the inulin (DP = 21) and ~8% of the nystose and 1-kestose were converted by TK1-FEH, sucrose was unaffected (Figure 3c).

Furthermore, less inulin was converted when inulin and sucrose were used as combined substrates, suggesting that sucrose acted as a dose-dependent inhibitor as shown for other plant 1-FEHs (Lothier et al., 2007; Xu et al., 2015) (Figure 3d). Compared to the activity displayed with inulin alone as the substrate, the presence of 8.8 mM sucrose reduced the conversion efficiency to 65%, and the presence of 88 mM sucrose reduced the conversion efficiency to 15%. These data, together with the lack of activity against sucrose as the sole substrate, confirmed that TK1-FEH is not an inverter. We thus propose that TK1-FEH is a functional 1-FEH with a substrate preference for inulin with a DP > 4.

**Overexpression of TK1-FEH results in the degradation of inulin**

The functional analysis of TK1-FEH was also carried out in planta by cloning the full-length TK1-FEH cDNA under the control of the constitutive CaMV35S promoter and expressing it in T. brevicorniculatum and T. koksaghyz. T. brevicorniculatum is apomictic and therefore produces genetically homogenous progeny, which facilitates the characterization of transgenic plants. Transformation resulted in more than 10 independent transgenic lines in both species.

Transgene expression in the T0 generation was confirmed by PCR, and quantitative analysis by real-time PCR (qPCR) revealed two transgenic T. brevicorniculatum plants (Tb1.2 and Tb4.2) and four T. koksaghyz plants (Tk1.7, Tk2.8, Tk3.1 and Tk8.1) with high, moderate or low levels of transgene expression (data not shown). These were used to generate T1 lines for subsequent analysis. Plants Tb1.2 and Tb4.2 were used to produce seven (Tb4.2) and nine (Tb1.2) T1 offspring, which were compared to nine wild-type T. brevicorniculatum control plants. The four transgenic T. koksaghyz plants (Tk1.7, Tk2.8, Tk3.1 and Tk8.1) were pollinated with one T. koksaghyz wild-type plant for seed generation, resulting in three (Tk1.7 and Tk8.1), four (Tk3.1) and six (Tk2.8) T1 plants for further investigation. The plants of both species were grown under greenhouse conditions and harvested after 18 weeks for the detailed analysis of development, gene expression and metabolite composition. Phenotypic analysis of the transgenic lines revealed that transgene expression had no impact on development in terms of biomass, flowering, seed setting or germination. The phenotypes of T1 plants from T. brevicorniculatum lines Tb1.2 and Tb4.2 are shown as representative examples in Figure 4a.

1-FEH gene expression was analysed in more detail by qPCR, revealing a significantly higher mean relative expression level in Tb1.2 (57-fold higher than the wild-type control) and Tb4.2 (sevenfold higher than the wild-type control) (Figure 4b). Transgenic T. koksaghyz lines Tk1.7, Tk2.8 and Tk8.1 expressed 1-FEH at high levels, and the transgenic near isogenic line (NIL) Tk3.1 was used as a control because the 1-FEH expression level was similar to wild type (Figure 4c). 1-FEH gene expression was 21.5-fold higher in line Tk1.7, 56.5-fold higher in line Tk2.8 and 68.3-fold higher in line Tk8.1, in each case compared to the control (Tk3.1). The quantitative analysis of 1-SST and 1-FFT gene expression revealed no significant differences between the transgenic lines and their controls, indicating that 1-FEH does not influence the transcription of fructosyltransferases in dandelion (Figure S3).

Although there were no phenotypic differences between transgenic lines and their controls, metabolic analysis revealed a significant reduction in the amount of inulin in the transgenic lines: 125 mg/g DW in line Tb1.2 and 274 mg/g DW in line Tb4.2 compared to 315 mg/g DW in wild-type plants (Figure 4d). Additionally, the average inulin DP in both T. brevicorniculatum lines differed significantly from the wild-type value of 11 FMs, that is four FMs in line Tb1.2 and nine FMs in line Tb4.2. T. koksaghyz lines Tk1.7, Tk2.8 and Tk8.1 also produced significantly lower amounts of inulin with lower average DPs than line Tk3.1 (Figure 4e). The amount and DP of inulin in all the T. brevicorniculatum and T. koksaghyz lines negatively correlated closely with the relative transgene expression level.

**Inulin degradation influences triterpene and NR levels in dandelion**

We next analysed in both dandelion species the increase in fructose and sucrose levels as a result of inulin degradation after 18 weeks. Both sugars increased in abundance in the transgenic T. brevicorniculatum and T. koksaghyz lines compared to control lines, mirroring the 1-FEH expression levels (Figure 5a and b). The amount of sucrose in lines Tb1.2 and Tb4.2 was 48 and 24 mg/g DW, respectively, compared to the wild-type level of 16 mg/g DW. In the transgenic T. koksaghyz lines, the sucrose level reached a maximum of 27 mg/g DW (line Tk8.1) compared to 15 mg/g DW in the control line Tk3.1. The fructose levels in both species were affected in a similar manner (Figure 5a and b). The free sugar levels could potentially promote among other pathways the biosynthesis of different isoprenoid end products in the roots, such as triterpenes and NR. Therefore, we determined the quantity of sterols, pentacyclic triterpenes and poly(cis-1,4-isoprene) in T. brevicorniculatum and T. koksaghyz (Table 1, Figure 5c and d). GC-MS analysis revealed significantly higher levels of sterols (campesterol, stigmasterol and sitosterol) and a remarkable increase in pentacyclic triterpenes such as taraxasterol, taraxerol, α-amyrin, β-amyrin and lupeol, in all the transgenic lines compared to the corresponding controls.

The quantity of poly(cis-1,4-isoprene) correlated in both species with the 1-FEH expression level. In T. brevicorniculatum line Tb1.2, the amount of poly(cis-1,4-isoprene) was almost 10 mg/g DW, compared to ≤ 4 mg/g DW in wild-type plants (Figure 5c). In contrast, although the average quantity of poly(cis-1,4-isoprene) also increased in line Tb4.2, the increase compared to wild-type plants was not statistically significant (4.8 mg/g DW). These data concur with the intermediate level of 1-FEH gene expression and the lower impact on inulin and sugar (fructose and sucrose) levels in those plants (Figures 4b, d and 5a). T. koksaghyz produces up to 10 times more NR in the roots than T. brevicorniculatum, as shown by the poly(cis-1,4-isoprene) content of 32.6 mg/g DW in control line Tk3.1. Notably, the overexpression of 1-FEH increased the quantity of poly(cis-1,4-isoprene) even in the transgenic T. koksaghyz lines, reaching a maximum mean value of 80 mg/g DW in line Tk8.1 (Figure 5d). Furthermore, we found that the amount of poly(cis-1,4-isoprene) correlated with the expression levels of two genes encoding enzymes in the rubber transferase pathway engineering 745

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Figure 4 Overexpression of Tk1-FEH in dandelion leads to inulin degradation. Investigations were carried out using root material from 18-week-old T. brevicorniculatum (n = 6–9) and T. koksaghyz (n = 3–6) plants (T1 generation). Asterisks indicate statistically significant differences between transgenic plant lines and their controls (* P ≤ 0.05; ** P ≤ 0.01; and *** P ≤ 0.001; unpaired t-test or Mann–Whitney U test). (a) Eight-month-old T. brevicorniculatum plants overexpressing Tk1-FEH (Tb1.2 centre; Tb4.2 right) and their control (Tb-WT left) (scale bar = 2 cm). (b and c) 1-FEH mRNA levels in roots of T. brevicorniculatum (b) and T. koksaghyz (c) plants determined by qPCR and normalized using the housekeeping genes TbEf1α and TkEf1α. (d and e) Inulin content and mean DP in T. brevicorniculatum (d) and T. koksaghyz (e) plants determined by HPLC.
complex (CPT and RTA) and the rubber elongation factor gene (REF) encoding a rubber particle-stabilizing protein (Epping et al., 2015; Laibach et al., 2015). A significant increase in the expression of CPT, RTA and REF individually or together was observed in lines Tb1.2, Tk2.8 and Tk8.1 compared to the control plants (Figure 5e and f).
In addition to 18-week-old plants, we analysed 40-week-old *T. brevicorniculatum* plants overexpressing *Tk1-FEH* to investigate the metabolic impact of inulin degradation at the late harvesting stage in a normal cultivation period. As above, none of the transgenic plants showed any phenotypic aberrations (data not shown), but we observed *1-FEH* dosage-dependent differences in the inulin, poly(cis-1,4-isoprene) and triterpene levels compared to wild-type plants (Figure 6 and Table S2). In contrast to 18-week-old plants, a further increase in the rubber content was evident solely in line Tb4.2 and not in line Tb1.2 (Figures 5c and 6e). This may reflect the fact that the inulin content in 18-week-old Tb1.2 plants was already low (~125 mg/g DW, Figure 4d) due to the strong expression of *1-FEH* (Figure 4b). In contrast, the inulin content in line Tb4.2 (~275 mg/g DW; Figure 4d) was only minimally affected compared to wild-type plants due to the low level of *1-FEH* expression (Figure 4b). Therefore, the inulin pool in line Tb4.2 can still be converted into rubber as indicated by the increase in the rubber content of 40-week-old plants (Figure 6e). Additionally, a significant portion of the inulin appeared to be metabolized for other housekeeping functions, reducing the inulin content of the 40-week-old wild-type plants by ~100 mg/g DW (Figure 6b) compared to 18-week-old plants (Figure 4d). This was also evident in the transgenic lines. The de novo biosynthesis of inulin in older plants appears unlikely due to the low level of the enzymes 1-SST and 1-FFT (Figure 4c). The 3'-RACE PCR coding sequences were amplified from root cDNA using the Universal Genome-Walker™ Kit (Clontech, Saint-Germain-en-Laye, France) according to the manufacturer’s instructions, with 1-FEH-GW1 and 1-FEH-GW2 as gene-specific oligonucleotides (Table S3).

### Amplification of full-length 1-SST, 1-FFT and 1-FEH cDNA sequences

The 1-SST and 1-FFT coding sequences were amplified from *T. brevicorniculatum* and *T. koksgahyz* root cDNA using the oligonucleotide combinations 1-SST-Sall-fwd/1-SST-NheI-rev and 1-FFT-NotI-fwd/1-FFT-XbaI-rev (Table S3), respectively. All oligonucleotides were based on cDNA sequences from *T. officinale* 1-SST (AJ250634) and 1-FFT (AJ829549), as well as *T. officinale* 1-FFT-specific EST data (DY802367). The Tb1-FEH andTk1-FEH coding sequences were amplified from root cDNA by 3'-RACE PCR using 1-FEH-GSP1-3' based on *T. officinale* EST data (DY815781) as the gene-specific oligonucleotide (Table S3). The 3'-RACE PCR was carried out as previously described (Schmidt et al., 2010a). The partial 1-FEH sequences were completed by genome walking using the Universal Genome-Walker™ Kit (Clontech, Saint-Germain-en-Laye, France) according to the manufacturer’s instructions, with 1-FEH-GW1 and 1-FEH-GW2 as gene-specific oligonucleotides (Table S3).

### Total RNA extraction and cDNA synthesis

Total RNA was extracted from the roots of wild-type *T. koksgahyz* plants using the NucleoSpin® RNA Plant kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s instructions. Total root RNA from other *Taraxacum* plants was extracted using the innuPREP RNA Mini Kit (Analytik Jena, Jena, Germany) according to the manufacturer’s instructions. The cDNA was synthesized using the all-in-one PrimeScript™ Reverse Transcriptase Master Mix from TAKARA (Clontech) according to the manufacturer’s instructions.

### Gene expression analysis by quantitative real-time PCR

Quantitative real-time PCR (qPCR) was carried out as previously described (Laibach et al., 2015). *T. koksgahyz* wild-type samples (harvested from May to November 2013) represented nine technical replicates of three individual cDNAs synthesized from overall higher yield per acre in autumn. Furthermore, we found that the inulin and NR contents in *T. koksgahyz* are influenced not only by changes in *Tk1-FEH* gene expression during the growing season, but also by the basal expression level, leading to natural variation in rubber productivity in different dandelion accessions. Our data indicate that the inulin content and 1-FEH expression level are important biochemical and genetic markers that can be used to select rubber-enriched annual *T. koksgahyz* varieties that can be used as a commercially feasible crop for future industrial NR production.

### Experimental procedures

#### Plant material and cultivation

*Taraxacum koksgahyz* wild-type plants, used for the analysis of inulin and NR metabolism throughout the growing season, were sown early in March 2013 and cultivated as previously described (Laibach et al., 2015). Early in May, the young plants were placed outside the greenhouse with supplemental irrigation. On day 15 of every month, three plants were picked randomly, and the roots were harvested, quick-frozen, lyophilized and ground to powder. Plants used in the *Tk1-FEH* overexpression experiments were sown in the greenhouse under the conditions described by Laibach et al. (2015). The plants were harvested 18 or 40 weeks after sowing, and the roots were processed as described above.
Figure 6  Effect of Tk1-FEH overexpression in 40-week-old T. brevicorniculatum plants. Investigations were performed with root material from 40-week-old T. brevicorniculatum (n = 5) plants (T1 generation) grown in the greenhouse. Asterisks indicate statistically significant differences between transgenic plant lines and their wild-type control (*P ≤ 0.05; **P ≤ 0.01; and ns = nonsignificant; Mann–Whitney U test). The 1-FEH (a), Tb-1-SST and Tb1-FFT (c) and TbCPT, TbRTA and TbREF (f) mRNA levels in roots of T. brevicorniculatum plants determined by qPCR and normalized using the housekeeping gene TbEf1a. (b) Inulin content and mean DP in T. brevicorniculatum plants determined by HPLC. (d) Fructose (HPLC) and sucrose (1H-NMR) levels in T. brevicorniculatum plants. (e) Poly(cis-1,4-isoprene) levels (1H-NMR) in T. brevicorniculatum plants (f) Relative TbCPT, TbRTA and TbREF mRNA levels in roots of T. brevicorniculatum plants determined by qPCR and normalized using TbEf1a.
one total root RNA sample of three pooled plants. Samples for all other qPCRs represented three technical replicates of root material from one individual plant, later pooled as 3–9 biological replicates. The housekeeping gene elongation factor 1a (Ef1a) was used for the normalization of gene expression in T. brevicorniculatum (TbEF1a) and T. koksaghyz (TkEF1a). Oligonucleotides used to measure gene expression levels are listed in Table S3. Conserved sequences were used for each oligonucleotide so that they were suitable for both T. brevicorniculatum and T. koksaghyz. Quantitative PCR data were analysed as previously described (Laibach et al., 2015). Species- and root-specific oligonucleotide efficiencies were calculated as previously reported (Table S4).

Cloning the Tk1-FEH overexpression constructs

The full-length Tk1-FEH cDNA was amplified using oligonucleotides 1-FEH-Xhol-fwd and 1-FEH-XbaI-rev (Table S3) and inserted into the expression vector pLab12.10 using the restriction sites Xhol and XbaI (Xing et al., 2014). The final construct (pLab12.10-CaMV35S-Ptk1-FEH-CaMV35ST) was validated by sequencing.

Agrobacterium-mediated transformation of Taraxacum spp.

The transformation of T. brevicorniculatum and T. koksaghyz plants was carried out as previously described with slight modifications (Post et al., 2012). T. koksaghyz leaf discs were incubated on callus induction medium containing 400 mg/L amoxicillin, and shoot induction medium was supplemented with 1 mg/L kinetin, 100 μg/mL indole acetic acid and 200 mg/L amoxicillin. Root induction in T. koksaghyz was triggered by placing the shoots on shoot induction medium supplemented with 400 mg/L amoxicillin.

Analysis of inulin levels and DP in dandelion roots by HPLC

Ground root material was boiled for 18 h at 85 °C using HPLC-grade water (1:10 v/w) as the solvent. For clarification, the inulin-containing extract was centrifuged at 5000 g for 20 min, 500 l of the supernatant was incubated with 500 l 20 mM acetate buffer (pH 4.15) for 2 h at 55 °C, shaking at 700 r.p.m. Another 500 l of the supernatant was incubated with 490 l 20 mM acetate buffer (pH 4.15) and 10 l (110 μL/L) Aspergillus niger inulinase (Sigma-Aldrich, St. Louis, MI) dissolved in the same buffer. Both reactions were stopped by adding 1 l EDTA (pH 8.0), followed by centrifugation at 13 000 g for 2 min. To verify the activity of inulinase, we also digested 20 mg/mL chichory inulin dissolved in HPLC-grade water. Fructose, glucose and sucrose levels in each sample were determined by comparing the fructose, glucose and sucrose standards used for calibration and to establish detection limits. The inulin content was determined by comparing the fructose, glucose and sucrose levels of undigested and digested samples of the same inulin extract as previously described with slight modifications (Hahn et al., 2016).

The mean DP of inulin was determined by considering sucrose as the smallest possible sugar molecule resulting from the degradation of inulin either by 1-FEH or by inulinase. Therefore, sucrose was not considered in the determination of the mean DP, which was calculated as previously reported (Hahn et al., 2016).

Determination of poly(cis-1,4-isoprene) levels by 'H-NMR spectroscopy

Poly(cis-1,4-isoprene) levels were analysed by 'H-NMR using 150–200 mg of ground root material supplemented with 1500 μL of a mixture containing 10% toluene-d8, tetramethylsilane and 16 mm 2,6-dimethoxyphenol (DMOP) as internal standards. Extraction was carried out for 16 h at 20 °C, shaking at 1000 r.p.m. After centrifugation (21 000 g, 110 min), 600 l of the supernatant was analysed using a Bruker Avance III 400 MHz spectrometer with a 5-mm broadband inverse (BB) probe head (Bruker, Billerica, MA) at 298 K. All data were acquired using a one-dimensional 'H-NMR pulse program with 90° pulse and a relaxation delay of 20 s. The raw data were processed including the correction of the phase and baseline. For quantitative analysis, the C5 methyl signal for poly(cis-1,4-isoprene) was integrated at 1.75 ppm and the methyl signal of DMOP at 3.34 ppm. The quality control for each run was performed by checking the integrals of DMOP against calibrator samples. A control sample with a known poly(cis-1,4-isoprene) content was also analysed in each run.

Determination of sucrose levels by 'H-NMR spectroscopy

Sucrose was extracted from 100 mg of ground root material by adding 1500 μL 0.1 mM phosphate buffer (pH 6.8) containing 5% D2O and 1 mM trimethylsilylpropanoic acid (TSP) as an internal standard. Extraction was carried out for 1 h at 85 °C and 16 h at 20 °C, shaking at 1000 r.p.m., and 0.5 h at 85 °C again, followed by centrifugation (21 000 g, 10 min). Analysis of the supernatant (600 μL) was performed as described above at 310 K, with a 30° pulse and a relaxation delay of 15 s. Data analysis was carried out as above. The signal of the internal TSP standard integrated at 0.0 ppm and the signal at 5.44 ppm for sucrose (anomeric glucose proton) were used to quantify the sucrose content. Quality control was performed by comparing the integral of TSP against calibrator samples.

Extraction of triterpenes and GC-MS analysis

Triterpenes were extracted as described by Post et al. (2012) using 250 μg betulin as the internal standard. Hexane phases were pooled and evaporated to dryness, and the residue was dissolved in 1 mL acetonitrile overnight before analysis by GC-MS as described by Xing et al. (2014).

Heterologous expression in Pichia pastoris

The Tk1-FEH sequence was amplified from pLab12.10-CaMV35S-Ptk1-FEH-CaMV35ST using the oligonucleotide combination Tk1-FEH-fwd and Tk1-FEH-KpnI-rev (Table S3). The product was introduced into the pPink-HC vector at the Stul and KpnI sites, followed by transformation according to the manufacturer’s introductions (PichiaPink™ Expression System; Thermo Fisher Scientific, Darmstadt, Germany). Transgene integration was checked by colony PCR using the oligonucleotide combination 5’AOX and 1-FEH-RT-rev. Large-scale expression of the recombinant Tk1-FEH was achieved using PichiaPink™ strain 2, with untransformed and empty vector controls. Supernatants were separately frozen in liquid nitrogen and stored at −80 °C.
Purification and concentration of the recombinant Tk1-FEH from the supernatants were achieved using a combination of precipitation with 80% (v/v) ammonium sulphate and dialysis. The precipitated proteins were dissolved in 2 mL McIlvaine buffer (pH 6.0). The solution was dialysed against 1 L McIlvaine buffer (pH 6.0) at 4 °C for 18 h using a membrane with a 14 kDa cut-off. The dialysed samples were quick-frozen and stored at −20 °C before protein separation and visualization by SDS-PAGE (Laemmli, 1970), deglycosylation by Remove-iT™ PNGase F (New England Biolabs, Ipswich, MA) or enzymatic assays.

HPLC-coupled end point determination of Tk1-FEH characteristics

The end point enzymatic assays described below were followed by HPLC analysis to measure residual glucose, fructose and sucrose concentrations. The final substrate concentrations (mg/mL) were determined by the addition of the measured sugars plus one tenth of fructose. Based on these standards, the concentrations of fructose, glucose and sucrose were found to be 0.3–100 mg/mL. All reactions were stopped by enzyme denaturation at 80 °C for 5 min. After centrifugation (13 000 g, room temperature, 2 min), the samples were measured for HPLC as described above.

All assays were carried out using 5 µL of the purified supernatants containing 8.46 µg of total protein, determined using the Bradford method (Bradford, 1976). Control assays were prepared using supernatant from untransformed yeast, empty vector controls or yeast transformed with Tk1-FEH mixed with yeast transformed with empty vector controls or yeast transformed with Tk1-FEH mixed with Tk1-FEH. Conversion was determined after subtracting the empty vector control conversion.

Characterization of recombinant Tk1-FEH by mass spectrometry; pH-dependent HPLC-coupled end point determination of 1-FEH characteristics; Statistical analysis

See Supplementary methods.

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Conflict of interest

The authors declare no conflicts of interest.

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Supporting information
Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Partial multiple sequence alignment of several GH32-family proteins.

Figure S2. Characterization of recombinant Tk1-FEH.
Figure S3. 1-SST and 1-FFT gene expression levels in 18-week-old T. brevicorniculatum and T. koksaghyz plants overexpressing Tk1-FEH.
Table S1. Analysis of Tk1-FEH expression in yeast cultures by mass spectrometry.
Table S2. Triterpene content of 40-week-old T. brevicorniculatum plants overexpressing Tk1-FEH.
Table S3. List of oligonucleotides used in this study.
Table S4. Oligonucleotide efficiencies for qPCR.
Data S1. Supplementary methods.