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RESEARCH PAPER

The role of transketolase and octulose in the resurrection plant *Craterostigma plantagineum*

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Abstract

Phylogenetic analysis revealed that *Craterostigma plantagineum* has two transketolase genes (*transketolase 7* and *10*) which are separated from the other transketolase genes including *transketolase 3* from *C. plantagineum*. We obtained recombinant transketolase 3, 7, and 10 of *C. plantagineum* and showed that transketolase 7 and 10 of *C. plantagineum*, but not transketolase 3, catalyse the formation of octulose-8-phosphate *in vitro*. Transketolase 7 and 10 of *C. plantagineum* performed the exchange reaction that produces octulose-8-phosphate using glucose-6-phosphate and fructose-6-phosphate as substrates. Octulose is localized in the cytosol and phloem exudate analysis showed that octulose was the dominant sugar exported from the leaves to the roots.

Key words: Octulose, photosynthesis, resurrection plants, sugar transport, transketolase.

Introduction

D-Glycero-D-ido-octulose (octulose) is a rare monosaccharide which accumulates in the desiccation-tolerant plant *Craterostigma plantagineum* Hochst. The conversion between octulose and sucrose occurs when *C. plantagineum* undergoes dehydration and subsequent rehydration (Bianchi *et al.*, 1991). Considerable research has revealed that sucrose has a fundamental function in regulating osmotic potential and protecting membranes as well as macro-molecules in resurrection plants (Hoekstra *et al.*, 1997; Vicré *et al.*, 2004; Peters *et al.*, 2007; Dinakar and Bartels, 2013). Some efforts have been made to explain how sucrose is produced in *C. plantagineum* (Ingram *et al.*, 1997; Kleines *et al.*, 1999). In contrast to sucrose metabolism, the generation and physiological function of free octulose is still a mystery.

Octulose is an eight carbon monosaccharide, its monophosphate octulose-8-phosphate is an intermediate in the pentose phosphate pathway and may be synthesized via a novel alternative photosynthesis pathway (Flanigan *et al.*, 2006; Williams and MacLeod, 2006). Transketolase acts as a key enzyme in the pentose phosphate pathway and, in photosynthesis, it catalyses the formation of various sugar phosphates (Krüger and von Schaewen, 2003). It has been suggested to participate in octulose metabolism in *C. plantagineum*. Three isoforms of transketolase (tkt3, tkt7, and tkt 10) have been characterized at the molecular level in *C. plantagineum* (Bernacchia *et al.*, 1995). Transketolase extracted from rehydrated leaves of *C. plantagineum* catalysed the formation of octulose-8-phosphate using glucose-6-phosphate and β-hydroxypyruvate as substrates (Willige *et al.*, 2009). Similarly, Williams and MacLeod (2006) obtained octulose-8-phosphate in extracts from spinach through the exchange reaction with glucose-6-phosphate and fructose-6-phosphate as substrates. They proposed that the exchange reaction catalysed by transketolase is part of an alternative Calvin cycle (also called the alternative scheme). Although there is still a dispute about the exchange reaction, Clasquin *et al.* (2011) provided new evidence for this alternative scheme. They showed that D-glycero-D-altro-octulose-1,8-*bis*phosphate

might be synthesized in yeast by the aldol addition of dihydroxyacetone phosphate and ribose-5-phosphate which was catalysed by the glycolytic enzyme fructose *bis*phosphate aldolase. This is consistent with another part of the alternative scheme proposed by Flanigan *et al.* (2006). Therefore, it is necessary to examine whether the exchange reaction is performed by transketolase isolated from *C. plantagineum*. Storage and transport are important components of sugar metabolism which are involved in carbon allocation in plants. The appearance of octulose in the roots of *C. plantagineum* (Norwood *et al.*, 2003) raises the question, could octulose be transported?

Transketolase protein was extracted from *C. plantagineum* leaves and the recombinant *C. plantagineum* transketolases 3, 7, and 10 were obtained from the corresponding over-expression construct. Proteins were used to test the exchange reaction. GC/MS analysis demonstrated that transketolases 7 and 10 can perform the exchange reaction and octulose was synthesized. Octulose is accumulated in the cytosol, acting as an excellent antioxidant (Zhang and Bartels, 2016). Octulose is also exported from the leaves to the roots in *C. plantagineum*.

Materials and methods

Plant material

C. plantagineum plants were grown as previously described by Bartels *et al.* (1990).

Gene cloning and protein purification

The cDNA fragments encoding the *C. plantagineum* genes *TKT3*, *TKT7*, and *TKT10* were amplified by PCR and cloned into the PJET1.2 vector according to the manual of molecular cloning (Sambrook *et al.*, 2001), the guide to the CloneJET PCR cloning kit (Thermo scientific, #k1231), and the Thermo scientific protocol of DNA digestion and ligation. Detailed information on the gene sequences of *TKT3*, *TKT7*, and *TKT10* of *C. plantagineum* is given by Bernacchia *et al.* (1995). Enzyme-digested fragments were ligated to the digested pET28a+ vector and transformed into the *Escherichia coli* expression strain BL21 (DE3). All of the primers used were designed by software Oligo 7 and their sequences are listed in Supplementary Table S1 at JXB online. Protein expression and purification were conducted according to Kirch and Röhrig (2010). Purified proteins were dissolved in the elution buffer: 50 mM HEPES/NaOH (pH 7.4), 300 mM NaCl, 250 mM imidazole, 10% (v/v) glycerol, 0.1% (v/v) Triton X-100, and 1.5 mM β-mercaptoethanol. The empty vector pET 28a+ was used as the control in the transformation and protein purification from *E. coli* BL21 (DE3) cells. Protein concentrations were determined according to Bradford (1976) using the Bio-Rad kit (Bio-Rad Laboratories, Inc. USA).

Transketolase extraction from plant tissue and protein analysis

Transketolase from plant leaves was purified according to Bernacchia *et al.* (1995). The pellet that was obtained after 50–70% (w/v) $(NH_4)_2SO_4$ precipitation was dissolved in 500 µl buffer A (50 mM TRIS–HCI, pH 7.5, 10% (v/v) glycerol, 10 mM $MgCl_2$) and used for enzymatic assay.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) of the proteins was conducted according to Laemmli (1970). Protein Western blot analysis was performed as described by Bartels *et al.* (1991) and the transketolase antiserum was prepared by Bernacchia *et al.* (1995).

Enzymatic assays and product dephosphorylation

The enzymatic reactions were performed as described by Willige *et al.* (2009): 25 µg purified protein, 58 mM glycylglycine (pH 7.7), 0.01% (w/v) Na-azide, 0.002% thiamine pyrophosphate, 15 mM $MgCl_2$, 5.3 mM acceptor substrate (ribose-5-phosphate or glucose-6-phosphate), and 16 mM donor substrate (β-hydroxypyruvate or fructose-6-phosphate).

After the catalysing reaction, sugar phosphates were dephosphorylated according to Willige *et al.* (2009). The dephosphorylated products were purified by passing through a column containing ion-exchange bed resin AG 501-X8(D) (BIO-RAD). The flow-through fractions were used for GC/MS analysis.

Carbohydrate extraction and sugar analysis by GC/MS

Sugars were extracted from plant tissues as described by Willige *et al.* (2009). Freeze-dried plant material was ground to a fine powder and extracted twice with 80% (v/v) methanol (3 ml g^{-1}) at 4 °C. The homogenates were cleared by centrifugation for 5 min at 5 000 *g* and 4 °C. The methanol was evaporated to dryness under reduced pressure at 25 °C. The sediment was taken up in water and washed three times with chloroform to remove lipophilic substances. The aqueous phase was centrifuged for 30 min at 10 000 *g* and 4 °C to remove any particles. A cation (Dowex 50 WX8) and an anion (Dowex IX8) exchange resin were added to the aqueous phase (5 g resin per 100 ml) to remove organic acids, amino acids or other charged molecules. After stirring for 1 h the aqueous phase was transferred into a reaction vial for GC analysis.

The extracted sugar fractions were further separated and identified by coupled gas chromatography (GC)/flame ionization detection (GC/FID) and coupled gas chromatography/mass spectrometry (GC/MS). Ten µl of extract, prepared as described above, and 10 µg of xylitol (used as the internal standard) were dried at 60 °C under N_2 gas. 30 µl pyridine and 30 µl N,O-Bis(trimethylsilyl)-trifluoracetamide (BSTFA) were then added and the sample was diluted with 50 µl chloroform to reach a mass between 1 ng and 20 ng. The samples were heated for 40 min at 70 °C. The trimethylsilyl (TMS) sugar derivatives were separated on a DB1 column (J&W Scientific, Folsom, CA, USA). Qualitative GC/MS analysis was carried out with a gas chromatograph 7890B, detector 5977A MS Detection HP (Agilent Technologies, Böblingen, Germany); quantitative analysis was carried out with GC/FID (5890Series II Plus, HP, Agilent Technologies) (Willige *et al.*, 2009). One µl of each sample was injected and the H_2 flow was set to 37 kpa. The initial temperature was 65 °C for 3 min, after which the temperature was raised at a rate of 8 °C min^{-1} to a temperature of 240 °C, after which the rate was increased by 12 °C min^{-1} to a final temperature of 310 °C for 35 min. Data analysis was performed with GC ChemStation [Rev.B.03.02(341), Agilent Technologies, Böblingen, Germany].

Chloroplast isolation

Chloroplasts of *C. plantagineum* were isolated according to Rowan and Bendich (2011). Plant leaves were rinsed briefly with 70% (v/v) ethanol, followed by distilled water. Four grams of *C. plantagineum* leaves were mixed into 100 ml XPI solution (50 mM HEPES pH 7.5, 330 mM sorbitol, 5 mM ascorbic acid, 1 mM $MgCl_2$, 1 mM $MnCl_2$, and 2 mM EDTA) and homogenated on ice using a blender. The homogenate was filtered through three layers of Miracloth. The filtered liquid was centrifuged at 4 °C at 3 000 *g* for 5 min. The pellet was suspended in 5 ml XPI solution and layered on to the prepared 30/70% Percoll gradient (70% Percoll at the bottom, 30% Percoll on top). The mix was centrifuged at 4 °C at 12 000 *g* for 10 min using a swing-out rotor. The purified chloroplasts were collected from the band at the interface between the 30% and 70% Percoll layers using glass Pasteur pipettes. The chloroplasts were washed twice with 10 vols of XPI solution and centrifuged at 12 000 *g* for 20 s. Finally, the isolated chloroplasts were freeze-dried and kept at –70 °C.

Thin layer chromatography (TLC) and octulose purification

Sugars were separated by TLC silica gel 60 (CAS 105553, Merck Millipore) using *n*-butanol:H_2O:*n*-propanol (9:1:0.5 by vol.) as the developing solution for about 2 h. The plate was then dried at 37 °C and the sugars were detected by spraying the plates with a mixture of EtOH:H_2SO_4:HAc:anisaldehyde (90:5:1:5 by vol.) and heating to 100 °C for 5 min (Kutzer, 2004).

The area containing the sugars was excised from the silica gel and saturated in ethanol overnight. After centrifuging at maximum speed for 10 min and filtration through a 40 µm filter membrane, the ethanol solution was evaporated to dryness under reduced pressure at 25 °C. Finally, the syrup was weighed and dissolved in water for GC/MS analysis.

Phloem exudate analysis

The analysis was performed according to Wingenter *et al.* (2010). At the end of the illumination, the petiole ends of mature leaves from 6-week-old plants were cut under water and then transferred quickly into reaction tubes containing 100 µl of 15 mM EDTA solution (pH 7.25). The sugar export experiment was done in a water-saturated atmosphere overnight. The solution was then heated to 95 °C for 3 min in the reaction tubes before being cooled down for GC analysis (5 mM $CaCl_2$ inthe reaction tubes was used as a control). To increase the sugar concentration for the GC analysis, the leaf exudate solutions were condensed to about 20 µl by evaporating under reduced pressure at 25 °C. The sugar content is presented as a percentage of the total sugars.

Phylogenetic analysis of transketolase genes

Transketolase sequences were retrieved by Blast from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/) and obtained from the RNA sequencing of *Lindernia brevidens* and *Lindernia subracemosa* (unpublished data). The phylogenetic tree was generated from aligned sequences of predicted proteins from 51 plant transketolase genes by Maximum Likelihood Analysis using 2 000 bootstrap predictions and the 50% majority rule in MEGA6 (Hall, 2013).

Results

Molecular phylogeny of plant transketolases

Phylogeny analysis of the transketolase genes demonstrates that TKT7 and TKT10 are closely related in *C. plantagineum* and have diverged from other transketolase genes of desiccation-sensitive species while TKT3 shares a higher identity with other plant species (Fig. 1). Using RNA sequencing, we have found eight possible homologous genes for TKT3, TKT7, and TKT10 of *C. plantagineum* in another two octulose-producing plants *L. brevidens* and *L. subracemosa* (Phillips *et al.*, 2008). In the phylogenetic tree, TKT7 and TKT10 and their five homologues from *L. brevidens* and *L. subracemosa* (con 8 lb, con 3 ls, con 2 lb, con 1 lb, and con 5 ls) form a separate branch. Three homologues from *L. brevidens* and *L. subracemosa* (labelled as con 6 LS, con 1 LS, and con 2 LS) show highly similar evolutionary characteristics with TKT3 in another branch. The branch with *C. plantagineum* TKT3 appears closer to the big group that is composed of transketolases from another 48 angiosperm species. This shows the specificity of the TKT7 and TKT10 genes in octulose-producing plants in evolution.

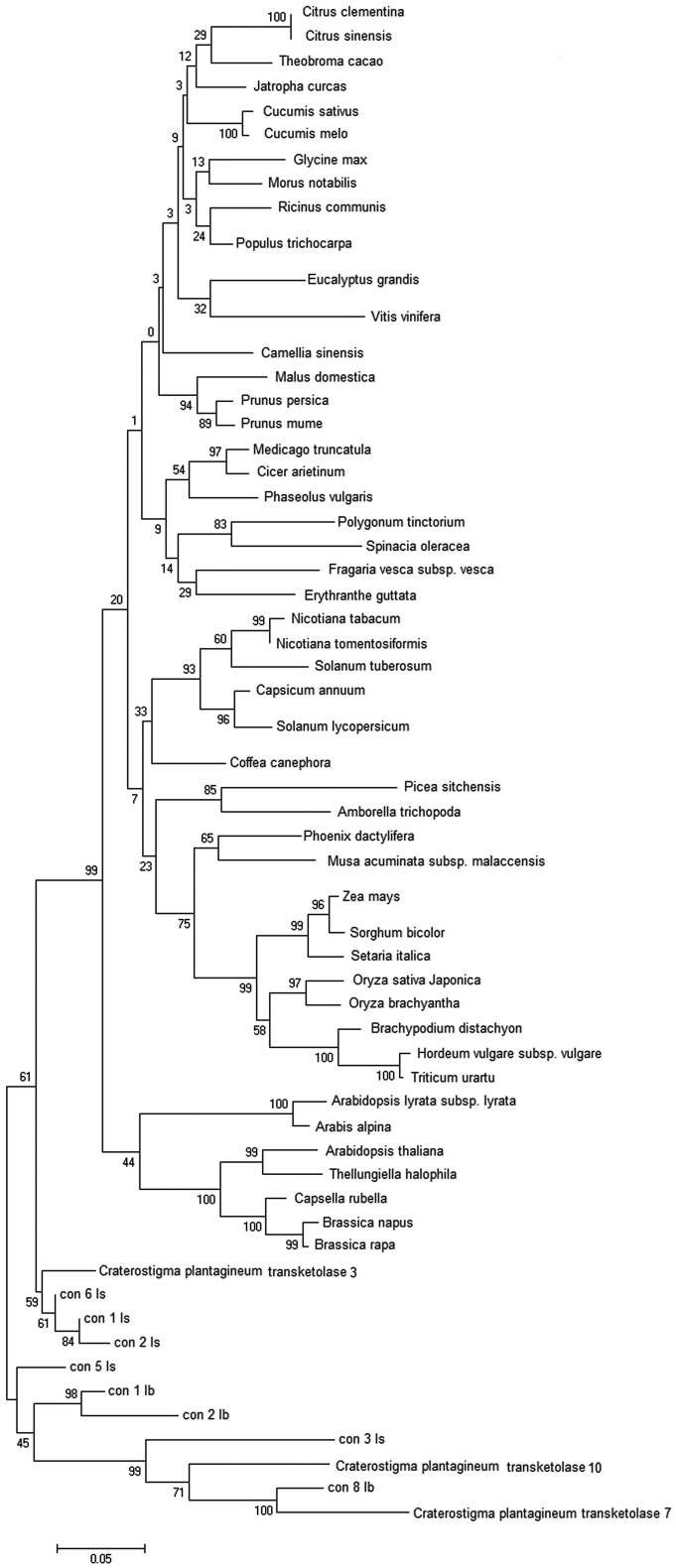

Fig. 1. Phylogenetic tree of plant transketolases constructed by Mega 6 software using amino acid sequences obtained from NCBI databases and the transcriptomes of *Lindernia brevidens and Lindernia subracemosa*. The homologues of transketolase genes from *L. brevidens* are con 1 lb, con2 lb, and con 8 lb and from *L. subracemosa* are con 1 ls, con 2 ls, con 3 ls, con 5 ls, and con 6 ls.

In vitro *synthesis of octulose*

Protein extracts enriched in transketolase were obtained from leaves of *C. plantagineum* by fractionated ammonium sulphate precipitation. Immunoblotting showed that transketolase was concentrated in the fractions precipitated by 50–70% (w/v) ammonium sulphate (Fig. 2). This transketolase

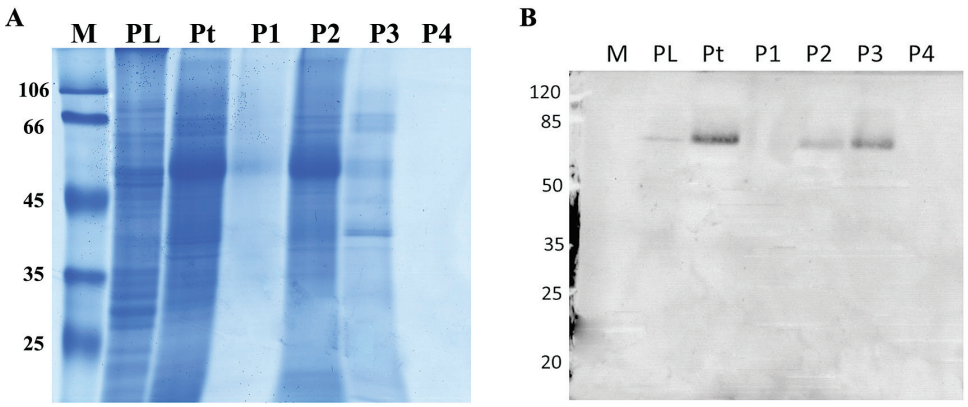

Fig. 2. SDS-PAGE (a) and immuno blotting (b) of proteins extracted from leaves of *C. plantagineum*. Note: M, protein size markers (given in kDa); PL, proteins not soluble in extraction buffer; Pt, total proteins extracted by the extraction buffer; P1, proteins precipitated by 0–25% (w/v) $(NH_4)_2SO_4$; P2, proteins precipitated by 25–50% (w/v) $(NH_4)_2SO_4$; P3, proteins precipitated by 50–70% (w/v) $(NH_4)_2SO_4$; P4, proteins remaining in extraction buffer after precipitation by 50–70% (w/v) $(NH_4)_2SO_4$; all pellets were dissolved in buffer A of the same volume as used in the extraction. Samples were loaded with SDS buffer on the gel.

containing protein fraction was used in reactions with glucose-6-phosphate and β-hydroxypyruvate as substrates. Octulose was detected in the reaction by verifying that its trimethylsilyl derivative has the same mass spectrum as that of the octulose standard purified from *C. plantagineum* leaves (Fig. 3A, B). Octulose-8-phosphate was synthesized in the reaction which confirmed a previous result that octulose-8-phosphate was synthesized (Willige *et al.*, 2009). Octulose-8-phosphate was also synthesized when β-hydroxypyruvate was substituted by fructose-6-phosphate (Fig. 3C). However, when ribose-5-phosphate was the acceptor substrate in the reaction, sedoheptulose-7-phosphate was formed, despite β-hydroxypyruvate or fructose-6-phosphate being the donor (Fig. 3 D, E).

The genes encoding transketolases 3, 7, and 10 were cloned in the expression vector pET28a+ and the corresponding recombinant proteins were expressed and purified from isopropyl β-D-1-thiogalactopyranoside-induced *E. coli* (BL21 DE3) cells. The quality and identity of the proteins was validated by SDS-PAGE (see Supplementary Fig. S1 at *JXB* online). Various reactions were carried out with the purified recombinant proteins and the dephosphorylated products of the reactions were analysed by GC/MS (Table 1). Results showed that transketolase 7 and transketolase 10 of *C. plantagineum* had the ability to catalyse the formation of octulose-8-phosphate using glucose-6-phosphate as acceptor and β-hydroxypyruvate or fructose-6-phosphate as donor, whereas transketolase 3 of *C. plantagineum* could not perform this function. However, all three recombinant proteins catalysed the formation of sedoheptulose-7-phosphate with ribose-5-phosphate as acceptor substrate and β-hydroxypyruvate or fructose-6-phosphate as donor. Neither octulose nor sedoheptulose were detected in the dephosphorylated products of reactions catalysed by the proteins purified from *E. coli* (BL21 DE3) cells transformed with the vector pET28a+.

These results indicate that the exchange reaction is feasible with a transketolase-enriched protein fraction from *C. plantagineum*. The recombinant *C. plantagineum* transketolases 7 and 10 also demonstrate the feasibility of the exchange reaction (Table 1). The differentiation of the catalysing functions of the three transketolase isoforms demonstrates that they have different catalytic properties which are connected with different roles in sugar metabolism in *C. plantagineum*.

Localization and transport of octulose

Although octulose accumulates to a high level, its cellular localization is as yet unknown. Therefore we attempted to identify the cellular compartment where octulose accumulates and how it is transported. Experimental results showed that the amount of octulose in isolated chloroplasts was lower than in whole leaf tissues in hydrated conditions or partially dehydrated conditions (Fig. 4). Partial dehydration led to a significant increase in total soluble sugars in leaf tissue tissues but it did not affect the levels of octulose and total soluble sugars in the chloroplast. This implies that octulose mainly accumulates in the cytosol.

Octulose was found to be the most abundant sugar in fully hydrated plants. However, a dedicated analysis showed that octulose levels fluctuate and are subject to a circadian rhythm (Supplementary Fig. S2). To explore the circadian change in octulose levels, phloem exudate analysis was conducted which demonstrated that 75–80% of the exported sugars is octulose (Fig. 5).

Discussion

As an important component of the photosynthesis reaction and the pentose phosphate pathway, the evolution of transketolase in plants might reflect the strategy of plants in adapting sugar metabolism to environmental requirements. Phylogeny analysis of transketolase genes demonstrates that TKT7 and TKT10 in *C. plantagineum* have diverged from the transketolase genes of many desiccation-sensitive species. The sequence analysis showed that *C. plantagineum* TKT7 and TKT10 and their homologous genes (e.g. con 8 lb from *L. brevidens* and con 3 ls from *L. subracemosa*) lack

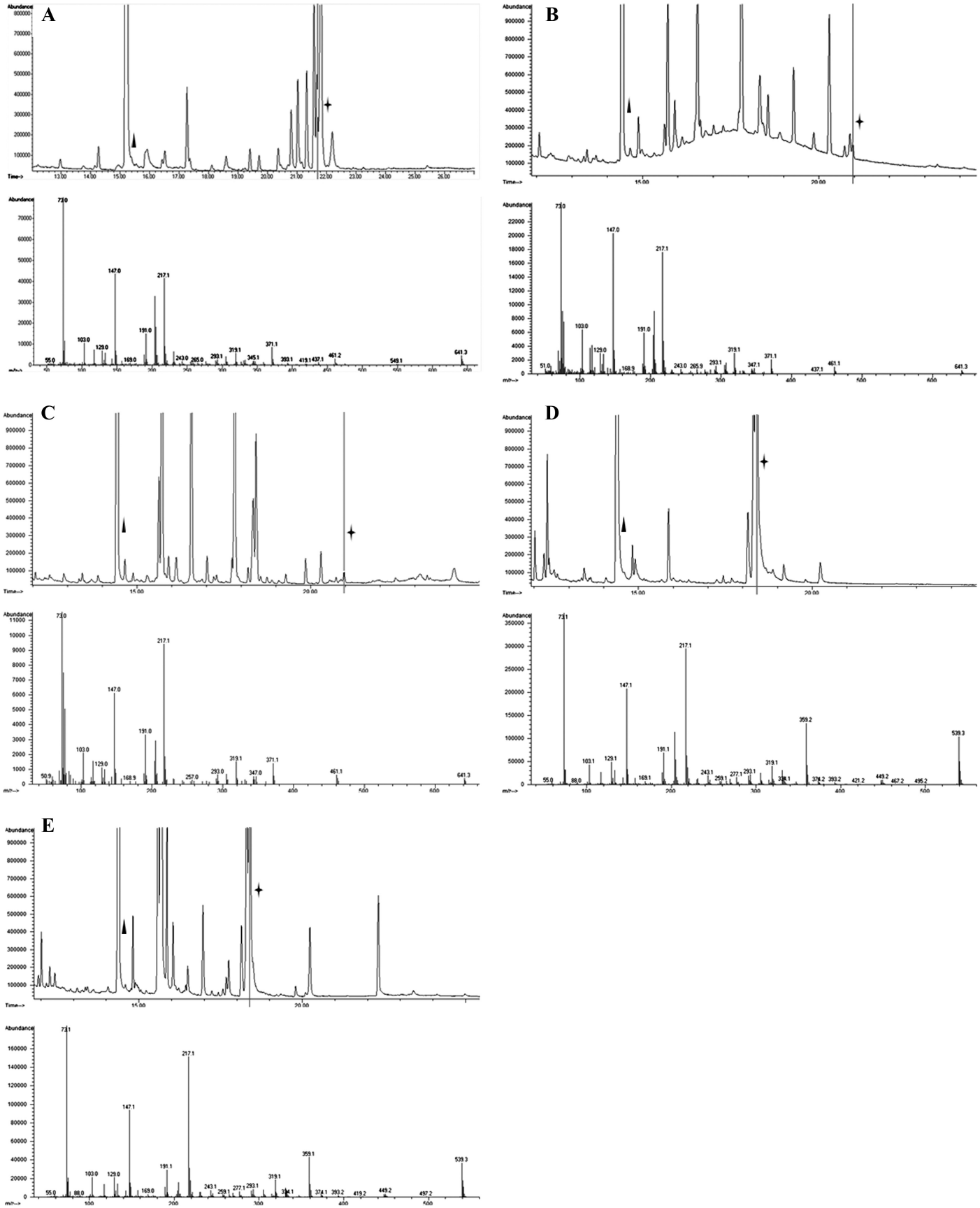

Fig. 3. (A) The chromatogram of the trimethylsilyl (TMS) derivatives of octulose purified from *C. plantagineum* leaves by TLC (upper part) and the mass spectra of the TMS derivative of octulose (lower part). The peak corresponding to the internal standard xylitol is labelled by a triangle and that of octulose is labelled by an asterisk. (B) The chromatogram of the TMS derivatives of the dephosphorylated reaction products with glucose-6-phosphate as acceptor substrate and β-hydroxypyruvate as donor (upper part) and the mass spectra of the TMS derivative of octulose (lower part). The peak

Table 1. *Summary of the dephosphorylated products obtained in transketolase-catalysed reactions in combination with different acceptor and donor substrates*

Enzyme[a]	Donor+acceptor HP+Glu-6-P[b]	Donor+acceptor HP+Rib-5-P[b]	Donor+acceptor Fru-6-P+Glu-6-P[b]	Donor+acceptor Fru-6-P+Rib-5-P[b]
CK	–	–	–	–
CKe	–	–	–	–
Tkt3	–	Sed[c]	–	Sed
Tkt7	Oct[c]	Sed	Oct	Sed
Tkt10	Oct	Sed	Oct	Sed
Tktp	Oct	Sed	Oct	Sed

[a] CK: reaction without protein; Cke: proteins purified from *E. coli* (BL21) transformed with vector pET28a+; Tktp: the protein enriched from leaf extracts of *C. plantagineum*.
[b] HP refers to β-hydroxypyruvate, Glu-6-P to glucose-6-phosphate, Rib-5-P to ribose-5-phosphate, and Fru-6-P to fructose-6-phosphate.
[c] Sed: sedoheptulose; Oct: octulose; –: no sedoheptulose or octulose found.

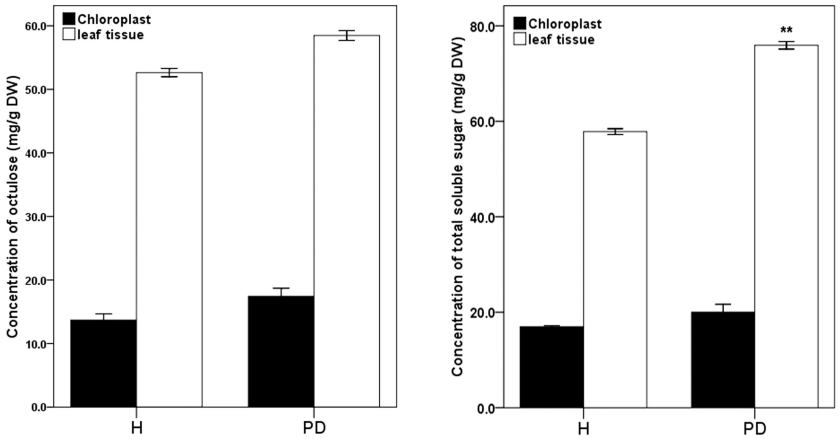

Fig. 4. The amounts of sugars in chloroplasts and leaf tissues in hydrated (H) and partially dehydrated (PD) conditions. In PD, plants were slowly dehydrated for 2 d when an RWC of 75% was reached in the leaves. All data represent means ±SD (n=3). Asterisks indicate significant differences determined with Student's t test (*P <0.05, **P <0.01).

recognizable transit peptides that transport the transketolase to the chloroplast. The transit peptide targeting transketolase to plastids is characteristic for most transketolase genes and is also present in TKT3 of *C. plantagineum* (Lange *et al.* 1998; Willige *et al.*, 2009). This may explain the diversification of *C. plantagineum* TKT7 and TKT10 and their homologous genes from *L. brevidens* and *L. subracemosa* and may be related to the fact that they encode enzymes involved in octulose metabolism in these plants.

Our results showed that TKT3 catalysed the transfer of a two-carbon ketol group from β-hydroxypyruvate or fructose-6-phosphate to ribose-5-phosphate to form sedoheptulose-7-phosphate. Besides catalysing the same transfer reaction, TKT7 and TKT10 also catalyse the formation of octulose-8-phosphate using glucose-6-phosphate as acceptor and β-hydroxypyruvate/fructose-6-phosphate as donor. This means that the exchange reaction is possible in *C. plantagineum* as it is in spinach (Williams and MacLeod, 2006). Willige *et al.* (2009) reported that TKT3 is localized in the chloroplasts while TKT7 and TKT10 are localized in the cytoplasm. Combined with the alternative Calvin cycle (Flanigan *et al.*, 2006), it is reasonable to suggest that TKT3 is only involved in

corresponding to the internal standard xylitol is labelled by a triangle and that of octulose is labelled by an asterisk. The reaction contains 25 μg purified protein, 58 mM glycylglycine (pH 7.7), 0.01% (w/v) Na-azide, 0.002% thiamine pyrophosphate, 15 mM $MgCl_2$, 5.3 mM donor, and 16 mM acceptor. After 24 h of the catalysing reaction, sugar phosphates in the product were dephosphorylated using acid phosphatase. The dephosphorylated products were purified on a column containing ion-exchanging bed resin AG 501-X8(D) (Bio-Rad). The flow-through fractions were used for GC/MS analysis. (C) The chromatogram of the TMS derivatives of the dephosphorylated reaction products with glucose-6-phosphate as acceptor substrate and fructose-6-phosphate as donor (upper part) and mass spectra of the TMS derivative of octulose (lower part). The peak corresponding to the internal standard xylitol is labelled by a triangle and that of octulose is labelled by an asterisk. The reaction conditions are the same as described in (B). (D) The chromatogram of the TMS derivatives of the dephosphorylated reaction products with ribose-5-phosphate as acceptor substrate and β-hydroxypyruvate as donor (upper part) and the mass spectra of the TMS derivative of sedoheptulose (lower part). The peak corresponding to the internal standard xylitol is labelled by a triangle and that of sedoheptulose is labelled by an asterisk. The reaction conditions are the same as described in (B). (E) The chromatogram of the TMS derivatives of the dephosphorylated reaction products with ribose-5-phosphate as acceptor substrate and fructose-6-phosphate as donor (upper part) and the mass spectra of the TMS derivative of sedoheptulose (lower part). The peak corresponding to the internal standard xylitol is labelled by a triangle and that of sedoheptulose is labelled by an asterisk. The reaction conditions are the same as described in (B).

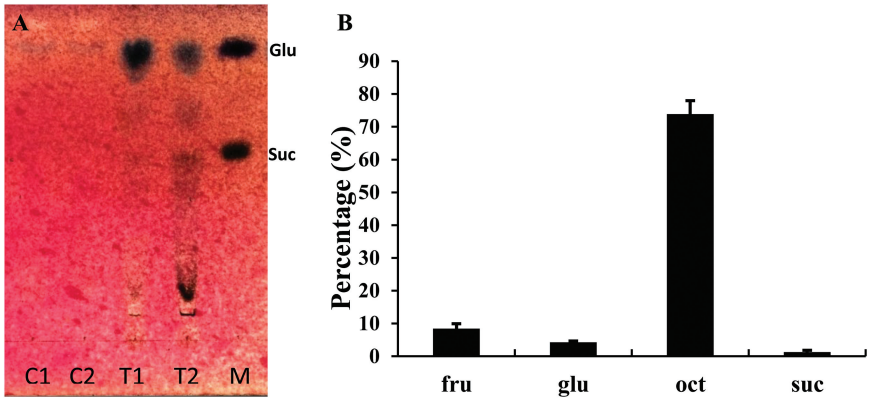

Fig. 5. Thin layer chromatogram (a) and GC analysis of leaf exudates (b) of *C. plantagineum*. C1 and C2 are two replicates of the sugars exported into 5 mM $CaCl_2$ from leaves (control); T1 and T2 are two replicates of the sugars exported into 15 mM EDTA (leaf exudates); Lane M contains glucose (Glu) and sucrose (Suc) standards. The GC analysis shows the percentage of sugars relative to total sugars. All data represent means ±SD (n=3).

carbon reactions in photosynthesis and the pentose phosphate pathway, while TKT7 and TKT10 are responsible for the accumulation of octulose in *C. plantagineum*. TKT7 and TKT10 could also participate in the pentose phosphate pathway, as transketolases can accept various substrates (Williams *et al.*, 1987; Krüger and von Schaewen, 2003) which is also shown for both TKT7 and TKT10 in our study. Expression analysis of transketolase isoforms showed that *TKT3* is constitutively expressed in leaves and roots while *TKT7* and *TKT10* are up-regulated in leaves during rehydration of the desiccated plant (Bernacchia *et al.*, 1995). The up-regulation of *TKT7* and *TKT10* positively correlates with octulose accumulation during rehydration. This supports the notion that TKT7 and TKT10 catalyse the exchange reaction to produce octulose. However, we are still left with the question: Is the conversion from octulose to sucrose during rehydration achieved through the reverse exchange reaction or is there another pathway for the conversion? Although the transcript levels of TKT7 and TKT10 decrease during dehydration (Rodriguez *et al.*, 2010), dehydration treatments generally take more than 2 weeks compared with rehydration that is completed within 48 h. It is possible to achieve the conversion during the longer time period. It is also possible that octulose is converted to sucrose by another pathway that might comprise phosphotransferase, aldolase, triosephosphate isomerase, and fructose 1,6-*bis*-phosphatase as proposed by Williams and MacLeod (2006). Further experimentation is necessary to verify this hypothesis.

Except in *C. plantagineum*, octulose was also found to accumulate in several closely related Linderniaceae, such as *Lindernia brevidens*, *Lindernia subracemosa*, *Lindernia philcoxii*, *Lindernia numilarifolia*, *Lindernia exilis*, and *Lindernia acicularis* (Kutzer, 2004; Phillips *et al.*, 2008). Thus it can be speculated that the exchange reaction catalysed by transketolase is also present in these plant species. Similarly, the other isomers of octulose, such as D-glycero-Dmanno-octulose isolated from avocado and sedum species (Charlson and Richtmyer, 1960) and D-glycero-L-galacto-octulose isolated from *Persea gratissima*, *Sedum spectabile*, and *Primula oficinalis* (Sephton and Richtmyer, 1963; Begbie and Richtmyer, 1966), may also be synthesized by the exchange reactions catalysed by transketolase with substrates of specific stereo configurations.

Many monosaccharides do not occur naturally in the free state but are commonly found as phosphate-ester derivatives that are important intermediates in the breakdown and synthesis of carbohydrates in living organisms (Robyt, 1998). For some free monosaccharides, specific phosphatases exist, catalysing the dephosphorylation of their phosphate-ester derivatives, e. g. glucose and sedoheptulose (Van Schaftingen and Gerin, 2002; Ceusters *et al.*, 2013). It is likely that a phosphatase exists which dephosphorylates octulose-8-phosphate or its isomers to produce octulose in *C. plantagineum* or D-glycero-D-manno-octulose/D-glycero-L-galacto-octulose in *Persea gratissima*/*Sedum spectabile*.

Octulose was shown to be the dominant sugar in leaf exudates by phloem exudate analysis. This indicates that *C. plantagineum* leaves transport octulose to roots as an energy supply. Although, in general, sucrose is the main transport form in plants, some plants also transport raffinose and stachyose and/or sugar alcohols (Turgeon and Wolf, 2009). Some studies have also proposed sedoheptulose as the sugar transport form (Liu *et al.*, 2002; Ceusters *et al.*, 2013) and, therefore, octulose is a good candidate for sugar transport in *C. plantagineum* and related plants. Our study showed that octulose accumulation follows a circadian rhythm (Supplementary Fig. S2) as already indicated by Norwood *et al.* (2003). The circadian fluctuation in octulose levels might be explained by octulose transport or by the circadian regulation of biosynthetic enzyme activities.

Sugars are often seen as an energy resource and as signalling molecules in plant cells (Eveland and Jackson, 2012). In the present study, we compared octulose levels in isolated chloroplasts and intact leaf tissues which suggests that octulose is localized in the cytosol. It is also possible that octulose is stored in the vacuole which occupies >95% of the cell volume in fully turgid plants and could serve as sites for storing soluble carbohydrates. This hypothesis could not be proved, as a protocol for vacuole isolation for *C. plantagineum* could not yet be established. Additional experiments showed that partial dehydration did not affect octulose levels in the leaf tissues. This can be explained by the study of Kutzer (2004) which showed that, during dehydration, sucrose starts to be rapidly synthesized in *C. plantagineum* only when the RWC is

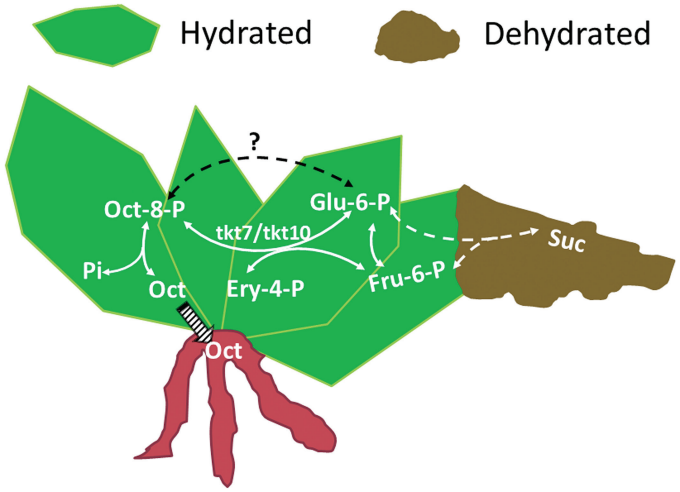

Fig. 6. A scheme of octulose metabolism in *C. plantagineum*. In hydrated conditions, octulose-8-phosphate (Oct-8-P) is synthesized through the exchange reaction that is catalysed by transketolase 7 (tkt7) and transketolase 10 (tkt10) using glucose-6-phosphate (Glu-6-P) and fructose-6-phosphate (Fru-6-P) as substrates. Oct-8-P is dephosphorylated to produce octulose (Oct). Oct can be transported from leaves to roots. In dehydrated conditions, Oct-8-P is transformed into Glu-6-P or Fru-6-P that is used to synthesize sucrose (Suc). Pi refers to phosphate. The striped arrow indicates the transport of octulose from leaves to roots. The dashed arrow only indicates the overall reaction direction (the details of the reaction are not shown).

below 75%. Similarly, Cooper and Farrant (2002) found that sucrose substantially accumulated in *Craterostigma wilmsii* only during the late stages of dehydration (below 25% RWC). Increasing evidence also suggests that sugar molecules counteract oxidative stress by acting as genuine ROS scavengers (Van den Ende and Valluru, 2009; Peshev *et al.*, 2013). Our analysis with the octulose isolated from TLC plates suggested that octulose has a stronger hydroxyl radical (OH^-) scavenging ability than sucrose (Zhang and Bartels, 2016). Previous studies showed that sucrose is a more efficient scavenger than glucose and fructose (Nishizawa *et al.*, 2008; Peshev *et al.*, 2013). The superior antioxidant properties of octulose might also be a reason for the high accumulation in *C. plantagineum*.

In conclusion, our study suggests that the *TKT7* and *TKT10* isoforms of transketolase show distinct specificity in function and evolution. They catalyse the synthesis of octulose-8-phosphate using glucose-6-phosphate and fructose-6-phosphate as substrate (Fig. 6). Octulose acts as a transport sugar in *C. plantagineum* and could play a role in antioxidant defence in the vacuole because of its stronger OH^- scavenging ability than other abundant sugars. To date, a scheme of the metabolism of octulose in *C. plantagineum* can be formed to explain the conversion between octulose and sucrose based on the cellular water status.

Supplementary data

Supplementary data can be found at *JXB* online.

Figure S1. SDS-PAGE of purified proteins: transketolase 3 (a), transketolase 7 (b), and transketolase 10 (c).

Figure S2. Diurnal variation of octulose levels in leaves of *C. plantagineum*.

Table S1. List of primers used in this study.

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References

Bartels D, Engelhardt K, Roncarati R, Schneider K, Rotter M, Salamini F. 1991. An ABA and GA modulated gene expressed in the barley embryo encodes an aldose reductase related protein. The EMBO Journal **10,** 1037.

Bartels D, Schneider K, Terstappen G, Piatkowski D, Salamini F. 1990. Molecular cloning of abscisic acid-modulated genes which are induced during desiccation of the resurrection plant *Craterostigma plantagineum*. Planta **181,** 27–34.

Begbie R, Richtmyer NK. 1966. The isolation of some heptoses, heptuloses, octuloses, and nonuloses from *Primula officinalis* Jacq. Carbohydrate Research **2,** 272–288.

Bernacchia G, Schwall G, Lottspeich F, Salamini F, Bartels D. 1995. The transketolase gene family of the resurrection plant *Craterostigma plantagineum*: differential expression during the rehydration phase. The EMBO Journal **14,** 610–618.

Bianchi G, Gamba A, Murelli C, Salamini F, Bartels D. 1991. Novel carbohydrate metabolism in the resurrection plant *Craterostigma plantagineum*. The Plant Journal **1,** 355–359.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry **72,** 248–254.

Ceusters J, Godts C, Peshev D, Vergauwen R, Dyubankova N, Lescrinier E, De Proft MP, Van den Ende W. 2013. Sedoheptulose accumulation under CO_2 enrichment in leaves of *Kalanchoë pinnata*: a novel mechanism to enhance C and P homeostasis? Journal of Experimental Botany **64,** 1497–1507.

Charlson AJ, Richtmyer NK. 1960. The isolation of an octulose and an octitol from natural sources: D-glycero-D-manno-octulose and DD-erythro-D-galacto-octitol from the avocado and D-glycero-D-manno-octulose from *Sedum* species. Journal of the American Chemical Society **82,** 3428–3434.

Clasquin MF, Melamud E, Singer A, *et al.* 2011. Riboneogenesis in yeast. Cell **145,** 969–980.

Cooper K, Farrant JM. 2002. Recovery of the resurrection plant *Craterostigma wilmsii* from desiccation: protection versus repair. Journal of Experimental Botany **53,** 1805–1813.

Dinakar C, Bartels D. 2013. Desiccation tolerance in resurrection plants: new insights from transcriptome, proteome, and metabolome analysis. Frontiers in Plant Science **4,** 482.

Eveland AL, Jackson DP. 2012. Sugars, signalling, and plant development. Journal of Experimental Botany **63,** 3367–3377.

Flanigan IL, MacLeod JK, Williams JF. 2006. A re-investigation of the path of carbon in photosynthesis utilizing GC/MS methodology. Unequivocal verification of the participation of octulose phosphates in the pathway. Photosynthesis Research **90,** 149–159.

Hall BG. 2013. Building phylogenetic trees from molecular data with MEGA. Molecular Biology and Evolution **30,** 1229–1235.

Hoekstra FA, Wolkers WF, Buitink J, Golovina EA, Crowe JH, Crowe LM. 1997. Membrane stabilization in the dry state. Comparative Biochemistry and Physiology Part A: Physiology **117,** 335–341.

Ingram J, Chandler JW, Gallagher L, Salamini F, Bartels D. 1997. Analysis of cDNA clones encoding sucrose-phosphate synthase in relation to sugar interconversions associated with dehydration in the resurrection plant *Craterostigma plantagineum* Hochst. Plant Physiology **115,** 113–121.

Kirch HH, Röhrig H. 2010. Affinity purification and determination of enzymatic activity of recombinantly expressed aldehyde dehydrogenases. Methods in Molecular Biology **639,** 282–291.

Kleines M, Elster R-C, Rodrigo M-J, Blervacq A-S, Salamini F, Bartels D. 1999. Isolation and expression analysis of two stress-responsive sucrose-synthase genes from the resurrection plant *Craterostigma plantagineum* (Hochst.). Planta **209,** 13–24.

Krüger NJ, von Schaewen A. 2003. The oxidative pentose phosphate pathway: structure and organisation. Current Opinion in Plant Biology **6,** 236–246.

Kutzer M. 2004. Untersuchung zum Zuckerstoffwechsel der Wiederauferstehungspflanze *Craterostigma plantagineum* und einiger Lindernia-Arten. PhD thesis, University of Bonn, Germany.

Laemmli U. 1970. Most commonly used discontinuous buffer system for SDS electrophoresis. Nature **227,** 680–685.

Lange MB, Wildung MR, McCaskill D, Croteau R. 1998. A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway. Proceedings of the National Academy of Sciences, USA **95,** 2100–2104.

Liu X, Sievert J, Arpaia ML, Madore MA. 2002. Postulated physiological roles of the seven-carbon sugars, mannoheptulose, and perseitol in avocado. Journal of the American Society for Horticultural Science **127,** 108–114.

Nishizawa A, Yabuta Y, Shigeoka S. 2008. Galactinol and raffinose constitute a novel function to protect plants from oxidative damage. Plant Physiology **147,** 1251–1263.

Norwood M, Toldi O, Richter A, Scott P. 2003. Investigation into the ability of roots of the poikilohydric plant *Craterostigma plantagineum* to survive dehydration stress. Journal of Experimental Botany **54,** 2313–2321.

Peshev D, Vergauwen R, Moglia A, Hideg E, Van den Ende W. 2013. Towards understanding vacuolar antioxidant mechanisms: a role for fructans? Journal of Experimental Botany **64,** 1025–1038.

Peters S, Mundree SG, Thomson JA, Farrant JM, Keller F. 2007. Protection mechanisms in the resurrection plant *Xerophyta viscosa* (Baker): both sucrose and raffinose family oligosaccharides (RFOs) accumulate in leaves in response to water deficit. Journal of Experimental Botany **58,** 1947–1956.

Phillips JR, Fischer E, Baron M, Van Den Dries N, Facchinelli F, Kutzer M, Rahmanzadeh R, Remus D, Bartels D. 2008. *Lindernia brevidens*: a novel desiccation-tolerant vascular plant, endemic to ancient tropical rainforests. The Plant Journal **54,** 938–948.

Robyt JF. 1998. *Essentials of carbohydrate chemistry*. Springer Science & Business Media.

Rodriguez MCS, Edsgärd D, Hussain SS, Alquezar D, Rasmussen M, Gilbert T, Nielsen BH, Bartels D, Mundy J. 2010. Transcriptomes of the desiccation-tolerant resurrection plant *Craterostigma plantagineum*. The Plant Journal **63,** 212–228.

Rowan B, Bendich A. 2011. Isolation, quantification, and analysis of chloroplast DNA. In: Jarvis RP, ed. *Chloroplast research in Arabidopsis*: *methods and protocols*, Vol. **1**. Humana Press: New York, 151–170.

Sambrook J, Russell DW, Irwin N. 2001. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY.

Sephton HH, Richtmyer NK. 1963. The isolation of a second octulose and of a heptose from the Avocado: D-glycero-L-galacto-octulose and D-glycero-D-galacto-heptose. The Journal of Organic Chemistry **28,** 1691–1694.

Turgeon R, Wolf S. 2009. Phloem transport: cellular pathways and molecular trafficking. Annual Revivew of Plant Biology **60,** 207–221.

Van den Ende W, Valluru R. 2009. Sucrose, sucrosyl oligosaccharides, and oxidative stress: scavenging and salvaging? Journal of Experimental Botany **60,** 9–18.

Van Schaftingen E, Gerin I. 2002. The glucose-6-phosphatase system. Biochemical Journal **362,** 513–532.

Vicré M, Farrant J, Driouich A. 2004. Insights into the cellular mechanisms of desiccation tolerance among angiosperm resurrection plant species. Plant, Cell and Environment **27,** 1329–1340.

Williams JF, Arora KK, Longenecker JP. 1987. The pentose pathway: a random harvest: impediments which oppose acceptance of the classical (F-type) pentose cycle for liver, some neoplasms and photosynthetic tissue. The case for the L-type pentose pathway. International Journal of Biochemistry **19,** 749–817.

Williams JF, MacLeod JK. 2006. The metabolic significance of octulose phosphates in the photosynthetic carbon reduction cycle in spinach. Photosynthesis Research **90,** 125–148.

Willige BC, Kutzer M, Tebartz F, Bartels D. 2009. Subcellular localization and enzymatic properties of differentially expressed transketolase genes isolated from the desiccation tolerant resurrection plant *Craterostigma plantagineum*. Planta **229,** 659–666.

Wingenter K, Schulz A, Wormit A, Wic S, Trentmann O, Hoermiller II, Heyer AG, Marten I, Hedrich R, Neuhaus HE. 2010. Increased activity of the vacuolar monosaccharide transporter TMT1 alters cellular sugar partitioning, sugar signaling, and seed yield in Arabidopsis. Plant Physiology **154,** 665–677.

Zhang Q, Bartels D. 2016. Physiological factors determine the accumulation of D-glycero-D-ido-octulose (D-g-D-i-oct) in the desiccation tolerant resurrection plant *Craterostigma plantagineum*. Functional Plant Biology (in press).