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The transketolase gene family of the resurrection plant *Craterostigma plantagineum*: differential expression during the rehydration phase

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Transketolases, key enzymes of the reductive and oxidative pentose phosphate pathways, are responsible for the synthesis of sugar phosphate intermediates. Here we report the first molecular analysis of transketolase genes from plants. Three distinct classes of transketolase-encoding cDNA clones were isolated from the desiccation-tolerant resurrection plant *Craterostigma plantagineum*. One class represented by the transcript *tkt3* is constitutively expressed in leaves and roots under all physiological conditions tested. By biochemical analysis and protein sequencing of purified transketolase, it was shown that *tkt3* is expressed in three enzymatically active isoforms. An intriguing discovery was that accumulation of the two other transketolase transcripts, *tkt7* and *tkt10*, is preferentially associated with the rehydration process of the desiccated plant; whereas *tkt10* is only expressed in leaves, *tkt7* was detected in leaves and roots. This observation suggests a possible role for these transketolases in the conversion of sugars, which are a major phenomenon in the rehydration process. Despite an abundant level of *tkt7* and *tkt10* transcripts in rehydrating leaves, proteins could not be isolated. This is due in part to a translational control mechanism acting on the loading of mRNAs to polysomes.

Key words: post-transcriptional control/rehydration/resurrection plant/sugar phosphates/transketolase

Introduction

The desiccation-tolerant plant *Craterostigma plantagineum* Hochst. (Family Scrophulariaceae) belongs to a small group of angiosperms whose vegetative tissues are able to tolerate severe dehydration (Gaff, 1971). Upon rewatering, the desiccated tissues rehydrate completely: the plants resume full physiological activities within hours and the changes caused by protoplasmic dehydration are reversed (Bartels *et al.*, 1990; Schneider *et al.*, 1993). This ability to withstand water loss is unique among higher plants and is shared only with lower plants (algae and bryophytes; Oliver, 1991), a few ferns (Reynolds and Bewley, 1993) and with specialized structures of vascular plants, like seeds, spores and pollen grains.

At the cellular level, drought stress is thought to cause cessation of plant growth (Hsaio, 1973) and general metabolism, together with irreversible damage to membrane structures and to the subcellular organization. The desiccation tolerance of poikilohydric plants implies that injuries produced by dehydration and the subsequent rewatering are minimized (Bewley and Krochko, 1982). It is assumed that molecules and compounds synthesized and accumulated during desiccation play an important role in the protection from stress. The biochemical and molecular basis for this protective mechanism is poorly understood.

Several genes relevant to desiccation have been characterized from *C.plantagineum* (Bartels *et al.*, 1990, 1992; Piatkowski *et al.*, 1990). The gene products accumulate abundantly in leaves and roots during dehydration, but they are rapidly degraded during the rehydration process (Bartels *et al.*, 1990; Bernacchia and Bartels, unpublished). Many of the isolated genes show homology with late embryogenesis abundant (*lea*) genes, which encode polypeptides constitutively expressed in seeds of higher plants during the later stages of embryogenesis (Skriver and Mundy, 1990; Bray, 1993); on the basis of the predicted structural features, Lea proteins are thought to contribute to osmoprotection (Dure *et al.*, 1989; Dure, 1993).

Besides the synthesis of proteins in *C.plantagineum*, major metabolic modifications of carbohydrates take place during the dehydration/rehydration cycle. The desiccation phase is characterized by a massive conversion of the dominant C_8 sugar in fully hydrated leaves, an unusual 2-octulose, into sucrose. During rehydration the sucrose level drops and octulose starts to accumulate again (Bianchi *et al.*, 1991). Sucrose synthesis in stressed *Craterostigma* leaves parallels the situation found in seeds of higher plants and in the desiccation-tolerant lower eukaryotes, where the content of specific sugars increases with tolerance acquisition (Koster and Leopold, 1988; Crowe *et al.*, 1984).

In *C.plantagineum* the accumulation of sucrose is accompanied by increases in the amount and in the activity of sucrose synthase and sucrose phosphate synthase; the induction of these enzymes is mainly regulated at the transcriptional level (Elster, 1994). Because of the observed conversions of sugars, a molecular approach was used to identify related changes in the gene expression pattern and hence to isolate genes specifically induced upon rewatering of dried *C.plantagineum* plants.

This approach resulted in the isolation of several transketolase-encoding genes. Transketolases (EC 2.2.1.1) are enzymes which occur ubiquitously in animals, plants and microorganisms (Racker, 1961); they catalyse the reversible transfer of an activated two-carbon glyco-

aldehyde moiety from a ketose to an aldose. Transketolases are key enzymes in the non-oxidative part of the pentose phosphate cycle and in the reductive Calvin cycle in photosynthetic organisms. In these pathways different sugar phosphate intermediates are synthesized, which can be channeled to carbohydrate metabolism or nucleic acid and amino acid biosynthetic reactions (Racker, 1961). Transketolase enzymes from animals, plants and yeast have been well characterized (Racker, 1961; Kochetov, 1982): they form homodimers made up of ~74 kDa subunits and they need thiamine diphosphate (ThDP) and divalent cations (preferentially Mg^{2+}) as cofactors during catalysis. Transketolase-encoding genes have been cloned from several microorganisms (*Rhodobacter sphaeroides*, Chen *et al.*, 1991; *Escherichia coli*, Sprenger, 1993; *Alcaligenes eutrophus*, Schäferjohann *et al.*, 1993; baker's yeast, Sundström *et al.*, 1993; *Hansenula polymorpha*, Janowicz *et al.*, 1985) and from human liver (McCool *et al.*, 1993), but not from plant species.

Here we report on a detailed analysis of transketolase genes isolated from the desiccation-tolerant plant *C.plantagineum* and on the purification of the transketolase enzyme. Three types of transcripts were distinguished, based on gene structure and expression patterns. Two classes of the transketolase transcripts identified were up-regulated during the rehydration process of dried *Craterostigma* plants.

Results

Transketolase-encoding cDNA clones tkt3, tkt7 and tkt10

A molecular analysis of the rehydration process in *Craterostigma* plants has revealed a class of transcripts specifically expressed in rehydrating tissues (manuscript in preparation). To isolate these transcripts, a cDNA library was constructed with poly(A)$^+$ mRNA isolated from fully rehydrated leaves (12 h rehydration). The library was screened with three different ^{32}P-labelled first strand cDNA probes derived from untreated leaves, dried and 12 h rehydrated leaves. cDNA clones hybridizing specifically to the rehydration-specific probe were selected for further analysis. One of these clones (*tkt7*) revealed a high sequence homology with transketolase-encoding genes. A further screening of the library resulted in the isolation of additional cDNA clones. On the basis of restriction enzyme analysis and cross-hybridization data, the available clones were assigned to the two groups, represented by *tkt7* and *tkt10*.

Amino acid sequencing of a purified transketolase enzyme indicated the existence of at least one additional transketolase-encoding gene in *Craterostigma*. To clone this gene, two degenerated oligonucleotides were designed representing the sequenced peptide and a highly conserved region of the protein (see Materials and methods and Figure 1). *Craterostigma* genomic DNA was then amplified in a polymerase chain reaction (PCR) and a fragment of the expected size was obtained. The sequence analysis confirmed the existence of a third class of *tkt* genes. A cDNA clone, not full-length, representing this third gene was then isolated from the initial cDNA library and its predicted amino acid sequence is shown in Figure 1 as *tkt3*, together with those of *tkt7* and *tkt10*. Because of its constitutive expression in all the tissues tested, including both desiccated and rehydrated tissues (see later), we will first describe the structure and characteristics of the *tkt3* genes.

The *tkt3* cDNA clone is closely related to transketolases across species. In particular 50 and 48% of the resulting Tkt3 amino acids were identical to those of transketolases from *A.eutrophus* (Schäferjohann *et al.*, 1993) and *E.coli* (Sprenger, 1993) respectively; 47% amino acid identity was found with the corresponding enzyme of the fungus *Pichia stipitis* (Metzger and Hollenberg, 1993) and of yeast (Sundström *et al.*, 1993). Moreover 45% of the amino acids of the *tkt3*-encoded polypeptide were found in the protein sequence of the transketolase of the photosynthetic prokaryote *Rhodobacter sphaeroides* (Chen *et al.*, 1991). In addition, the *tkt3* cDNA clone revealed homology to a formaldehyde transketolase from the methylotrophic yeast *Hansenula polymorpha* (37% amino acid identity; Janowicz *et al.*, 1985) and to a transketolase gene cloned from human liver (27% amino acid identity; McCool *et al.*, 1993). Like all the other transketolase-encoding sequences so far isolated, *tkt3* is similar to the *recP* gene isolated from *Streptococcus pneumoniae* (Radnis *et al.*, 1990); 50% identity is found at the DNA level and, if some frameshifts are added as suggested in Sundström *et al.* (1993), the predicted polypeptide shows a good level of identity with the Tkt3 protein (47%). Up to now, transketolase genes from plants have not been reported.

The predicted Tkt3 polypeptide shows a high sequence homology with the proteins encoded by the full-length cDNA clones *tkt7* and *tkt10* (see later). The predicted *Craterostigma* polypeptides and the protein sequences of enzymes from other organisms reveal highly conserved blocks of homology (see Figure 1). Those residues known to bind the cofactor ThDP and the C2 glycoaldehyde group during catalysis are perfectly conserved. These amino acids (marked by a star in Figure 1) have been identified by a crystallographic study of the yeast homodimeric enzyme bound to the cofactor (Lindqvist *et al.*, 1992). A comparative study on the ThDP binding site of several enzymes identified a consensus sequence for a particular secondary structure in the protein belonging to the cofactor binding domain (Hawkins *et al.*, 1989). For the transketolase enzymes this fingerprint is $GDG(X)_{8-10}$ $E(X)_4A(X)_4L(X)_7DXN$ (Reizer *et al.*, 1993): the consensus is found in the *Craterostigma* Tkt protein sequences (positions 175–197 in Tkt 7; see Figure 1). The crystallographic study on the yeast transketolase also identified some of the amino acids interacting with the backbone of the sugar substrate erythrose-4-phosphate during catalysis (marked with # in Figure 1; Sundström, 1992). Identical residues are found at the corresponding positions in the predicted *Craterostigma* Tkt polypeptides.

The tkt3 gene belongs to a small gene family and encodes constitutively expressed proteins which are enzymatically active

To specifically study *tkt3* genes, all the hybridization experiments were performed in conditions under which *tkt3* does not cross-hybridize with *tkt7* or *tkt10*. To analyse the organization of the *tkt3* genes, genomic DNA fragments of *Craterostigma* were hybridized with the insert of the

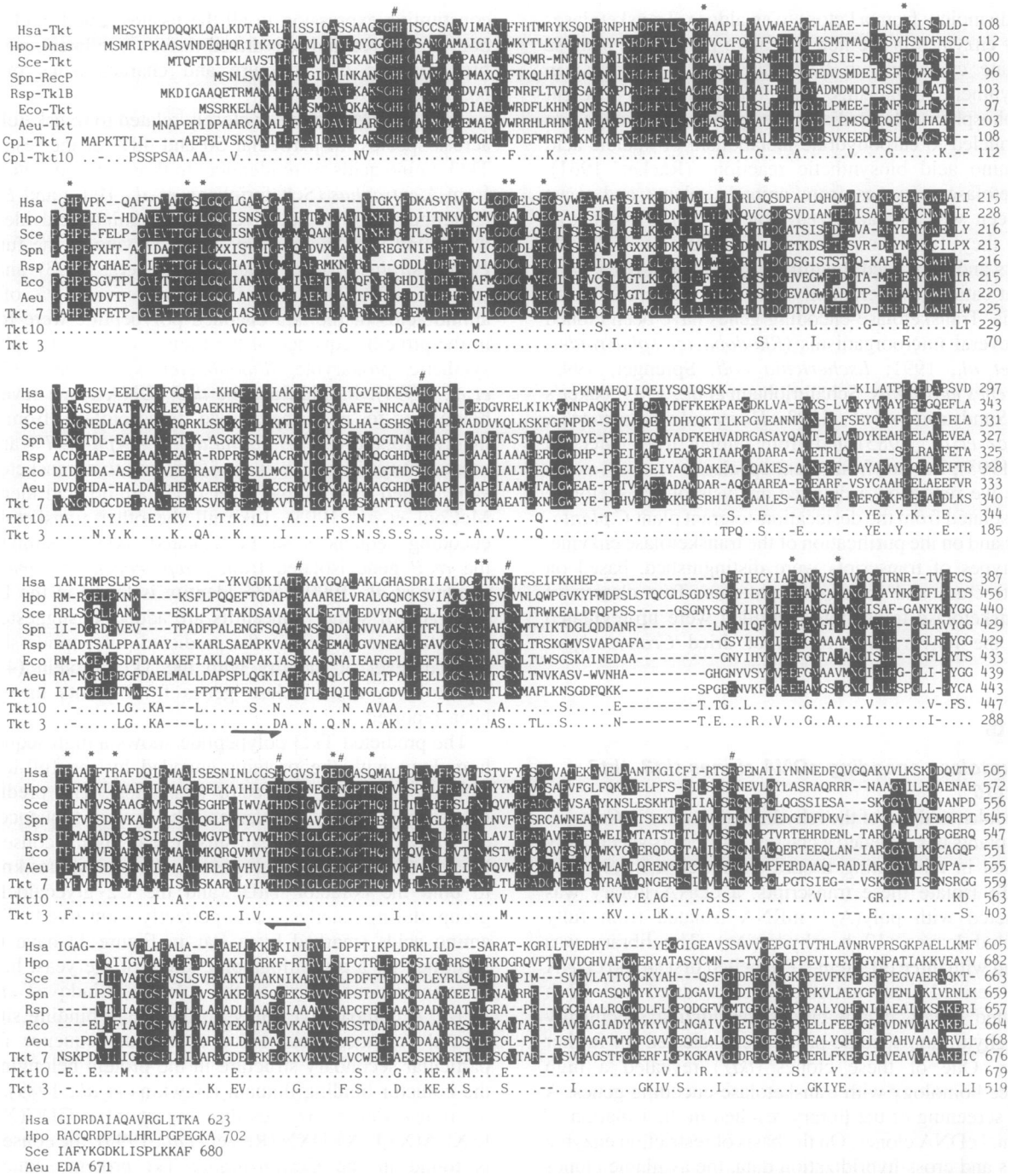

Fig. 1. Comparison of the deduced amino acid sequences of proteins (Tkt7, Tkt10 and Tkt3) encoded by three *Craterostigma tkt* genes with transketolases from other organisms. Hsa-Tkt, human transketolase protein (McCool *et al.*, 1993); Hpo-Dhas, dihydroxyacetone synthase amino acid sequence from *H.polymorpha* (Janowicz *et al.*, 1985); Sce-Tkt, *S.cerevisiae* Tkt I protein (Sundström *et al.*, 1993); Spn-RecP, predicted *S.pneumoniae* recP polypeptide (Radnis *et al.*, 1990) obtained via addition of some frameshifts denoted by an X as described in Sundström *et al.* (1993); Rsp-Tkt transketolase from *Rhodobacter sphaeroides* (Chen *et al.*, 1991); Eco-Tkt, transketolase encoded by the gene *tktI* from *E.coli* (Sprenger, 1993); Aeu-Tkt, *A.eutrophus* (Schäferjohann *et al.*, 1993). The Cpl-Tkt7 amino acid sequence is taken as an example of a full-length open reading frame. For Cpl-Tkt10 and Cpl-Tkt3, only the differences from Tkt7 are shown. Dashes are added to optimize the alignment. Arrows identify the PCR-amplified *tkt3* fragment. Residues interacting with the cofactor ThDP are marked by a star, while a # identifies those interacting with the sugar substrate. Amino acids identical between all three transketolases from *Craterostigma* and at least three of the other sequences are indicated in black. The DNA sequences have been assigned the following accession nos: *tkt3*, Z46646; *tkt10*, Z46647; *tkt7*, Z46648.

tkt3 cDNA clone, which gave rise to a few fragments in a genomic Southern blot (see Figure 2a). This implied the existence of a small *tkt3* gene family within the *Craterostigma* genome.

Expression studies of the *tkt3* genes (Figure 2b and c) only identified one band, of around 2200 nucleotides, in Northern blots. Hybridization experiments performed with RNA blots carrying samples from leaves and roots (Figure 2b and c) revealed a constitutive steady-state level of the transcript, without any appreciable differences between rehydrated, dried or untreated tissues. Thus it can be concluded that *tkt3* mRNA levels in *Craterostigma* leaves

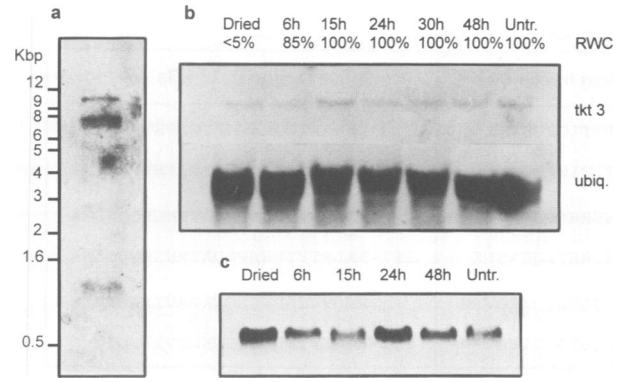

Fig. 2. The *tkt3* gene family. Southern blot analysis and expression patterns. (**a**) *Craterostigma* genomic DNA (10 µg), digested with *Hin*dIII, electrophoresed on an agarose gel and blotted onto a nylon membrane, was hybridized with the ^{32}P-labelled *tkt3* cDNA fragment. (**b**) Total RNA was extracted from dried, untreated (Untr.) or rehydrated *Craterostigma* leaf tissue (the time of rehydration is given in hours). The RWC of each sample considered is shown. RNA (30 µg) was fractionated by formaldehyde–agarose gel electrophoresis and blotted onto nylon membranes. After hybridization with the ^{32}P-labelled *tkt3* cDNA fragment the membrane was exposed for 48 h. To check that the RNAs were equally loaded, the filter was re-probed with a barley ubiquitin cDNA clone (Gausing and Barkardottir, 1986). (**c**) Total RNA (30 µg) extracted from roots during the rehydration process or from the two control tissues (dried or untreated) was tested for the presence of *tkt3* transcripts using the corresponding cDNA fragment as probe. After washing, the filter was exposed for 7 days.

Table I. Summary of the transketolase enzyme purification process

	Sp. act. (U/mg)	Total activity (U)	Purification (fold)
Crude extract	0.286	233	1
$(NH_4)_2SO_4$ precipitation (50–70% saturation)	0.947	183	3.3
Pooled DEAE–cellulose fractions	3.7	133	13
Mono-Q fraction I + II	20.95	52	73

and roots are not influenced by the tissue or its hydration state.

In order to relate the transketolase encoding genes of the *tkt3* family to transketolase enzymatic activity, active transketolase was isolated from untreated *Craterostigma* leaves. In this tissue, the level of transketolase enzyme is higher than in other tissues (see later). Most of the transketolase activity was obtained by precipitation of proteins from a crude extract at 50–70% (w/v) $(NH_4)_2SO_4$ saturation. Further purification was performed by ion exchange chromatography on a DEAE–cellulose column from which active transketolase was eluted with 0.1–0.15 M NaCl. This was followed by FPLC using a Mono-Q column; transketolase activity was recovered in two fractions (I and II) of the NaCl gradient between 0.1 and 0.2 M NaCl. The purification steps are summarized in Table I. The different fractions exhibiting transketolase activity were characterized by SDS–PAGE and Western blot analysis using an antiserum derived from a recombinant transketolase protein (Figure 3a and b). In fraction I the antibody recognized one band of 73 kDa and in fraction II two bands of 73 and 75 kDa (Figures 3 and 4). The proteins were eluted from these bands and partial

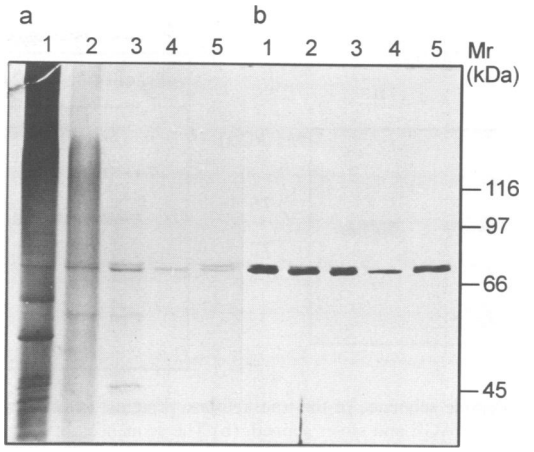

Fig. 3. Purification of the transketolase protein. Protein samples from the different purification steps were separated by 7.5% SDS–PAGE. One part of the gel was silver stained (**a**) and an identical second part was immunologically analysed in a Western blot (**b**). Lane 1, crude extract; lane 2, $(NH_4)_2SO_4$ precipitation pellet (50–75%); lane 3; pooled active DEAE–cellulose fractions; lane 4, Mono-Q Tkt fraction I; lane 5, Mono-Q Tkt fraction II.

peptide sequences were determined (Figure 4). All protein sequences could be assigned to the *tkt3* cDNA sequence. This indicates that all three purified enzyme fractions represent active Tkt3 proteins, thus confirming that the *tkt3* genes encode a small family of transketolases expressed in leaves and roots of *Craterostigma* plants.

The link between the enzymatically active transketolase enzyme and the DNA sequences was also established immunologically (Figure 5). The Mono-Q protein fractions I (Figure 5, lanes 1 and 3) and II (lanes 2and 4) were separated on a native gel which was then divided into two identical parts: one half (lanes 1 and 2) was stained for transketolase enzymatic activity and the other half (lanes 3 and 4) was blotted to nitrocellulose and immunologically analysed with the recombinant transketolase antiserum. The results of this experiment demonstrate that the enzymatic activity is assigned to the bands recognized by the recombinant antiserum.

Tkt3 transketolases have biochemical characteristics comparable with those of other transketolases (Racker, 1961). Gel filtration indicated that the native enzyme contained in the two pooled Mono-Q fractions was a dimer of ~140 kDa. Enzyme kinetics gave K_m values of 1.6 mM for ribose-5-phosphate (aldose in the enzyme assay) and 0.1 mM for xylulose-5-phosphate (ketose in the enzyme assay).

The data from this investigation of *tkt3* genes indicate that a gene family encoding active transketolase enzymes has been isolated and characterized in a plant organism.

Tkt7 and tkt10: genes highly expressed during rehydration

As pointed out when describing the isolation of *tkt* genes, transketolase cDNAs were initially discovered in a molecular analysis of the rehydration process. The two clones *tkt7* and *tkt10* were isolated as rehydration-specific probes from the cDNA library constructed with poly(A)$^+$ mRNA from 12 h rehydrated *Craterostigma* leaves.

Tkt7 and *tkt10* full-length open reading frames encode

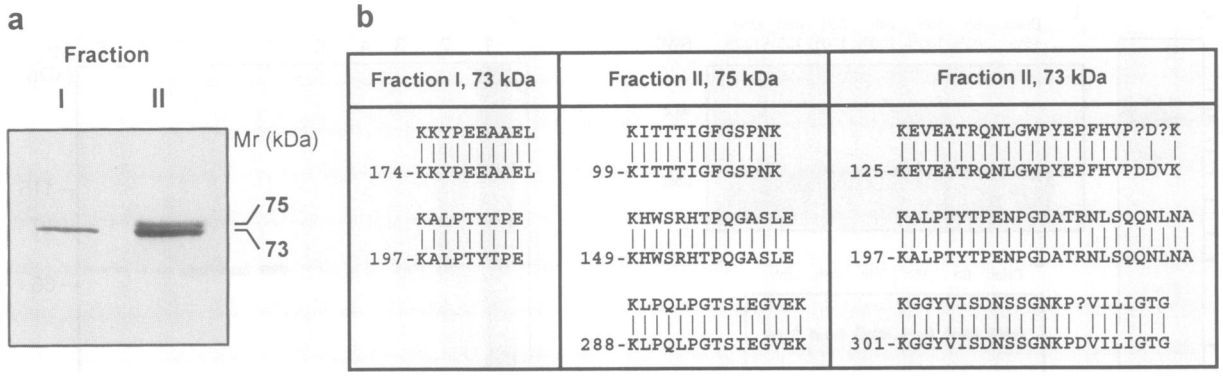

Fig. 4. Peptide sequence of the transketolase proteins. (**a**) The two Mono-Q protein fractions, I and II, with active transketolase were separated by 7.5% SDS−PAGE and silver stained. (**b**) The sequences of the peptides obtained from the different protein bands are aligned to the *tkt3*-encoded polypeptide sequence. Numbers refer to the positions given in Figure 1.

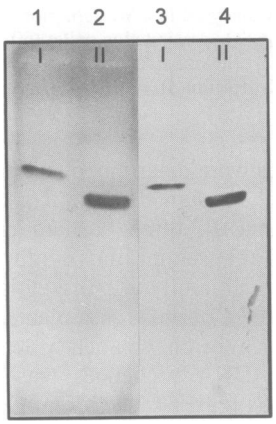

Fig. 5. Transketolase enzymatic activity stain and immunological analysis in protein gels. Two fractions (indicated as I and II) with active transketolase were obtained after the Mono-Q chromatography. After separation on a 7.5% native polyacrylamide gel these two fractions were analysed for transketolase using an enzymatic activity stain (lanes 1 and 2) and for immunological reaction with the recombinant transketolase antiserum (lanes 3 and 4). In both cases the same protein bands were identified. Lanes 1 and 3 correspond to fraction I and lanes 2 and 4 to fraction II.

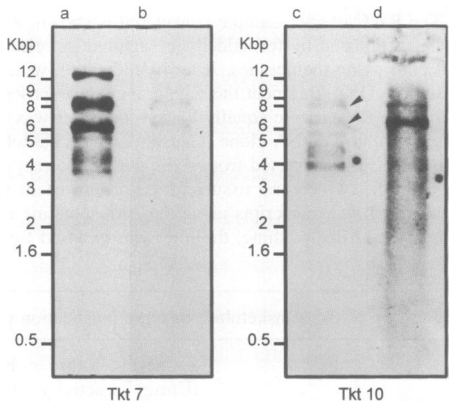

Fig. 6. DNA analysis of the *tkt7* and *tkt10* gene families in the *Craterostigma* genome. The DNA blot shown in Figure 2a was hybridized with the following probes: (**a**) full-length *tkt7* cDNA fragment; (**b**) *tkt7* 3′-specific oligonucleotide; (**c**) full-length *tkt10* cDNA fragment; (**d**) *tkt10*-specific oligonucleotide. The arrows in (c) point to those bands also detected in (d).

676 and 679 amino acids respectively, thus predicting proteins with a molecular weight of 74 kDa (Figure 1). Within the coding sequence, *tkt7* and *tkt10* show 80% homology at the nucleotide level and 93% identity for the predicted polypeptides. Both *tkt7* and *tkt10* were 78% homologous at the DNA level and 80% identical at the amino acid level to *tkt3*, but they are only partially homologous to the sequences of the peptides shown in Figure 4. The 3′-untranslated regions of the three sequences showed little homology; moreover, *tkt3* contains an extra 150 bp trailer not observed in the other two clones.

Like *tkt3*, clones *tkt7* and *tkt10* are representatives of small gene families (Figure 6). A Southern blot of *Craterostigma* genomic DNA was hybridized with *tkt7* and *tkt10* cDNA fragments and with gene-specific 50mer oligonucleotides designed from the 3′ ends of the two clones. Three bands were recognized by the *tkt7*-specific probe (Figure 6b), indicating that the *tkt7* family consists of three genes. The *tkt10*-specific probe (Figure 6d) hybridized with two DNA fragments, suggesting the presence of two copies of *tkt10* sequences in the *Craterostigma* genome.

The accumulation of the transketolase transcripts encoded by the *tkt7* and *tkt10* gene families was studied by Northern blot hybridizations (Figure 7). RNAs were extracted from differently treated leaf and root tissues of *Craterostigma* plants and hybridized with gene-specific 3′ probes. Figure 7a shows that the *tkt7* transcripts are preferentially accumulated during the rehydration process. They are first detected in leaves rehydrated for 6 h with a relative water content of 85%; the steady-state level of the transcript increases further, reaching a maximum in leaf tissues rehydrated for more than 15 h; after 48 h of rehydration a significant decrease is observed. In untreated fully hydrated leaves, a weak hybridization signal was observed for the *tkt7* transcript, while no signal was obtained from RNA of dried leaves.

The Northern blot hybridization performed with the *tkt10*-specific probe revealed that the steady-state level of expression of this gene reaches a maximum at the end of the water uptake period (after 15 h) and remains constant throughout the subsequent recovery period; after 48 h rehydration a slight decrease is observed, at which point the

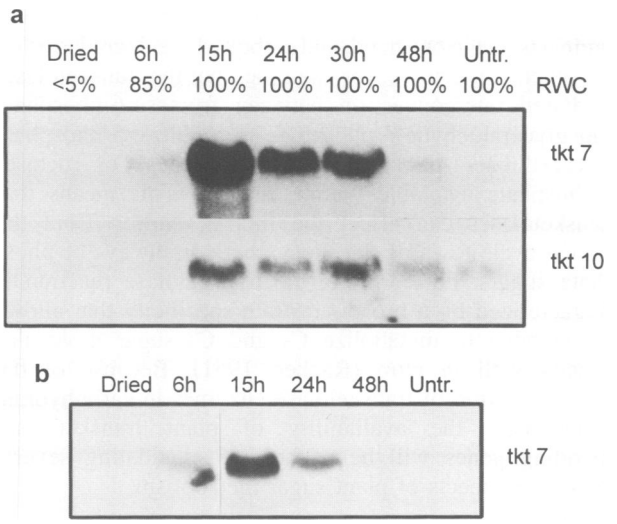

Fig. 7. Expression of *tkt7* and *tkt10* genes in *Craterostigma* tissues. (**a**) The same leaf RNA blot shown in Figure 2b was probed with ^{32}P-phosphorylated oligonucleotides specific for clone *tkt7* or *tkt10*. After washing, the membranes were exposed for 72 h. (**b**) *Tkt7* transcript accumulation in rehydrating roots of *Craterostigma* studied by hybridizing the RNA blot of Figure 2c with a *tkt7* radiolabelled probe.

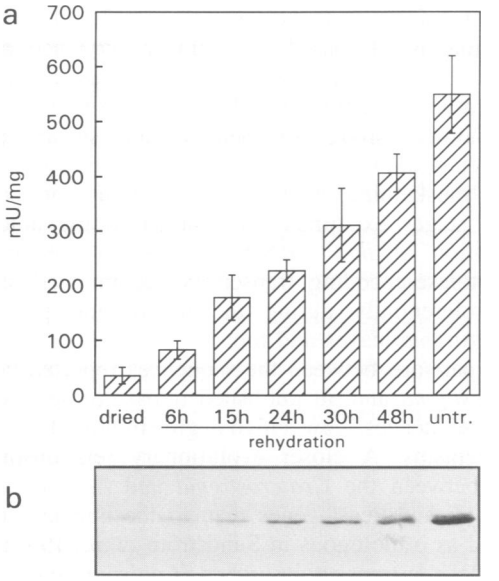

Fig. 8. Enzymatic activity of transketolase (**a**) and the corresponding Western blot (**b**) during rehydration of *Craterostigma* plants. Measurements were taken from dried leaves, rehydrated leaves (6, 15, 24 and 48 h rehydration) and untreated (untr.) fully hydrated leaves. The error bars indicate the standard deviation calculated for independent measurements from six different plants.

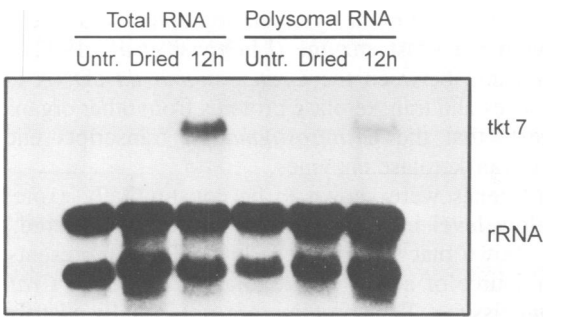

Fig. 9. Association of *tkt7* transcripts and polysomes during the rehydration process. Samples (20 µg) of total and polysomal RNA extracted from untreated, dried and 12 h rehydrated leaves were size fractionated and, after blotting onto nylon membranes, hybridized as indicated with a radiolabelled *tkt7* probe and, as a control, with a rRNA probe.

transcript level is indistinguishable from that of untreated *Craterostigma* leaves (Figure 7a).

tkt7 homologous transcripts were also detected in rehydrating roots of *Craterostigma* plants, with accumulation kinetics similar to those observed for leaves (see Figure 7b), except that no signal was obtained in the untreated tissue. In this tissue, *tkt10* transcripts were not identified with either the full-length cDNA probe or the 3′-specific oligonucleotide.

To analyse the expression of transketolases at the protein level during the rehydration process, polyclonal antibodies to *Craterostigma* were used. These antibodies were raised against a protein derived from a cDNA fragment which contained sequence blocks conserved between all three transketolase gene families. In coupled *in vitro* transcription/translation experiments it was shown that the transketolases derived from the cDNA clones were recognized by this antiserum. Total protein extracts prepared from untreated leaves, dried leaves and from leaves during the rehydration process were challenged with the transketolase antiserum and assayed for transketolase enzyme activity. Upon rewatering, the transketolase proteins increased in abundance, and the highest concentration was observed in untreated leaves (Figure 8a and b). In all samples, except for that from dried leaves, the amount of protein cross-reacting with transketolase antiserum directly correlated with the transketolase activity. In the dried leaves sample the enzymatic activity detected reached only half the value found in the 6 h rehydrated sample, although the amount of transketolase protein detected by the antibody in both samples was similar. This analysis revealed that accumulation of transketolase proteins during the rehydration process did not parallel the amount of *tkt7* and *tkt10* mRNAs revealed by Northern blot hybridizations.

The discrepancy observed between the increase in the steady-state level of *tkt7* transcripts and the accumulation of transketolase proteins in rehydrating tissues implied a reduced translation rate for this mRNA. To test this hypothesis, total and polysomal RNA fractions were extracted from differently treated *Craterostigma* tissues and hybridized with the *tkt7* homologous probe (Figure 9). The comparison of the relative abundance of this transcript in the two RNA populations revealed a reduced loading on polysomes in rehydrating tissues. It was concluded that *in vivo* only part of the *tkt7* mRNA was efficiently used for translation.

Discussion

This paper describes the molecular analysis of transketolase genes in *C.plantagineum*. Transketolase genes attracted our attention because they were identified as one of the few mRNAs up-regulated during the rehydration process in dried *Craterostigma* plants. Detailed analysis of the transketolases in *Craterostigma* revealed that three

different classes of transcripts (*tkt3*, *tkt7* and *tkt10*) can be distinguished, based on primary sequence and on distinct expression patterns. Southern blots indicate that each group is represented by two to three copies in the genome. Through biochemical analysis and protein sequencing, the *tkt3* class of mRNA was shown to encode active transketolase enzymes in all tissues and physiological stages examined. In contrast to the ubiquitous distribution of *tkt3* mRNA, it was notable that *tkt7* transketolase-encoding transcripts accumulated strongly and preferentially during the rehydration process in *Craterostigma* leaves and roots.

Comparisons between the sequences reported here for *Craterostigma* and all published transketolase DNA and protein sequences show a high degree of homology among all organisms. A closer evolutionary relationship was found between the *Craterostigma* and *A.eutrophus* and the *E.coli* and yeast genes than to the human sequence (defined as orthologous in Sundström *et al.*, 1993) or the formaldehyde transketolase from *H.polymorpha*, which is characterized by a different substrate specificity (Janowicz *et al.*, 1985). Significant amino acid identity is also found with the *recP* gene from *S.pneumoniae*, which was recently attributed to the transketolase protein family (Reizer *et al.*, 1993). The similarities between the predicted *Craterostigma* polypeptides and the related transketolase sequences include the residues which were identified as functionally important by crystallographic studies carried out with the yeast enzyme (Lindqvist *et al.*, 1992). The relationship between the *Craterostigma tkt* cDNA clone sequences and transketolase proteins from other organisms suggests that the *Craterostigma tkt* transcripts encode active transketolase enzymes.

tkt3 genes were shown to be constitutively expressed at a low level in all the tissue samples considered. We have shown that the corresponding mRNA represents the main source of active transketolase enzymes in *Craterostigma* tissues. Tkt3 proteins are not equally distributed in the plant tissues, but they reach the highest level in untreated leaves, from which active enzymes were purified. The observed difference in the accumulation profiles of *tkt3* mRNA and of the corresponding protein during the rehydration process suggests a slow turnover of *tkt3* protein and/or a high translation rate in untreated leaves. The purification and biochemical characterization of the active transketolase from *Craterostigma* leaves revealed some interesting features. After Mono-Q chromatography transketolase activity was attributed to three protein bands which could be identified by their amino acid sequences as belonging to the *tkt3* gene family. The different electrophoretic mobilities of the three Tkt3 transketolases can either be explained by post-translational modifications of the polypeptides or by the expression of several *tkt3* genes with differences in their N-termini which were not identified in this study. The ubiquitous distribution and the biochemical and structural characteristics of the *tkt3*-encoded proteins are consistent with their involvement in the general transketolase-dependent sugar metabolism taking place in the plant cell. As described in the Introduction, in plants transketolases play a key role in the Calvin cycle for carbon fixation and in the pentose phosphate pathway (Racker, 1961). Transketolase activity has a particular role in plant cells, since it creates a reversible link between sugars, amino acids and nucleic acid biosynthesis on the one hand, and carbohydrate degradation on the other. It is known, for example, that the transketolase-mediated interconversion between fructose-6-phosphate and glyceraldehyde-3-phosphate to xylulose-5-phosphate and erythrose-4-phosphate modulates the level of fructose-6-phosphate available within the cell. This means that transketolases can affect the flux of carbon from the Calvin cycle towards the biosynthetic pathways of phosphate sugars. Furthermore, the transketolase reaction is characterized by a broad substrate specificity that allows the enzyme to metabolize C_3 and C_7 sugar molecules equally well *in vitro* (Racker, 1961). Because of the versatile action of transketolase enzymes in carbohydrate metabolism, the availability of plant transketolase-encoding genes will be of help in elucidating several important aspects of plant sugar metabolism.

Tkt7 and tkt10 gene families: a translational control active during leaf rehydration?

Besides the characterization of the constitutively expressed *tkt3* gene family, we have shown that closely related transketolase-encoding transcripts are expressed in *Craterostigma* tissues. The primary structure of the related mRNAs, *tkt7* and *tkt10*, reveals features which allow them to be distinguished. *Tkt7* and *tkt10* are, with 93% identity, very similar to each other in their coding regions, suggesting that either these genes arose by duplication events or they reflect the high ploidy level characteristic of *Craterostigma* plants. However, the protein predicted from *tkt10* contains an extended serine- and alanine-rich N-terminus not found in related polypeptides. Furthermore, it showed no obvious homology with plant transit peptides (von Heijne *et al.*, 1991) and therefore it is uncertain whether this sequence contains subcellular localization signals. The constitutively expressed *tkt3*-encoded protein shares only 80% of amino acids with *tkt7* and *tkt10*; this suggests an early evolutionary divergence of the former sequence from the other two. The three transketolase transcripts possess specific 3′-untranslated regions. This divergence could reflect the absence of selective pressure on this part of the gene or it might even be important for mRNA stability or translational efficiency (Sullivan and Green, 1993).

The difference between the *Craterostigma tkt* gene families is even more evident at the level of their expression pattern. *tkt10* showed a leaf-specific expression, with its mRNA accumulating in the late phases of rehydration and in untreated leaves. Expression of the *tkt7* transcripts is directly correlated with the rehydration process in both leaves and roots. Interestingly, superimposed on this strict transcriptional regulation of the *tkt7* and *tkt10* gene families, an additional level of control must be hypothesized in order to explain the results obtained: Western blot analysis showed that the abundant accumulation of *tkt7* mRNA in rehydrating *Craterostigma* tissues is not reflected in a parallel increase in proteins.

In addition, although the *tkt10* gene family is expressed at high levels in untreated leaves, we have so far been unable to detect a corresponding protein by partial peptide sequencing. Possible explanations for this could include a translational control leading either to a low translation rate or to a marked instability of the encoded proteins. A

comparison between the total amount of *tkt7* mRNA and the fraction of *tkt7* transcripts bound to polysomes showed that only part of the mRNA was available for translation. A similar observation was made for pea ascorbate peroxidase mRNA during recovery from drought stress (Mittler and Zilinskas, 1994). In maize seedlings it was shown that during anaerobiosis the sucrose synthase transcript SS1, even if up-regulated and correctly loaded on polysomes, is subjected to a translational control, since the corresponding protein level did not change in the same way (McElfresh and Chourey, 1988). It is interesting that this type of control is linked to the special metabolic state of rehydrating *Craterostigma* leaves. Due to the relevance of Tkt proteins in drought-induced carbohydrate interconversions, the study of *tkt7* and *tkt10* gene expression in *Craterostigma* may allow a more precise approach to the metabolic basis of the resurrection phenomenon.

Materials and methods

Plant material

Craterostigma plantagineum (Hochst.) plants were grown in controlled growth chambers as described by Bartels *et al.* (1990). In each rehydration experiment, mature plants were allowed to dry in their pots by withholding water for a period of 9–10 days. Soil was then removed without destroying the roots and the plants were rehydrated by soaking them in water for the indicated periods of time of between 3 and 48 h. Tissues were detached and rapidly frozen in liquid nitrogen. The relative water content (RWC) of rehydrating tissues was measured as described previously (Bartels *et al.*, 1990) and was expressed as a percentage of the value of fully hydrated, untreated leaves.

Nucleic acid preparations

Total RNA was isolated from *Craterostigma* tissues according to De Vries *et al.* (1986), with the modification that two additional phenol and one chloroform/isoamylalcohol (100:1) extractions were made before the LiCl precipitation. Total polysomal RNA was isolated according to Forde *et al.* (1981). Poly(A)$^+$ mRNA was purified using oligo(dT) chromatography (Bartels *et al.*, 1990). *Craterostigma* genomic DNA was isolated as described by Doyle and Doyle (1990).

Nucleic acid separations, blotting and hybridizations

Northern and Southern blot analysis of RNA and DNA were performed as described in Bartels *et al.* (1990). Probes for the hybridizations were either DNA inserts corresponding to the different cDNA clones or, to discriminate between the two mRNA classes, 50 bp oligonucleotides derived from the 3′-untranslated regions of the *tkt7* and *tkt10* cDNA clones. In this case the hybridizations were performed for 16–18 h at 42°C in 6× SSC, 0.5% (w/v) SDS, 1× Denhardt's solution, 0.05% (w/v) sodium pyrophosphate and 100 µg/ml denaturated salmon sperm DNA. The filters were washed three times at 42°C in 4× SSC, 0.1% (w/v) SDS for 10 min and then exposed to X-ray films (Kodak X-Omat) with Trimax intensifying screens.

cDNA library construction and differential screening

mRNA of 12 h rehydrated leaves purified twice on oligo(dT)–cellulose was used as a template for cDNA synthesis. A lambda ZAP II (Stratagene) cDNA bank was constructed according to the supplier's instructions. Thirty thousand primary phages were plated and three replica filters were differentially screened with ^{32}P-labelled cDNA derived from the mRNAs of untreated leaves, dried leaves and dried tissue rehydrated for 12 h. Only phages hybridizing to the probe from rehydrated leaves were purified and the corresponding phagemids were isolated by *in vivo* excisions according to the manufacturer's protocol.

DNA sequencing and computer analysis

The isolated cDNA clones were sequenced on both strands, using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), using either the T7 DNA Polymerase Kit (Pharmacia) or the PRISMTM Ready Reaction Taq Dye-Deoxy Terminator Cycle Sequencing Kit (Applied Biosystems). In this case the reactions were resolved on an Applied Biosystems 373A DNA Sequencer. Computer alignments and nucleotide and amino acid sequence analysis were performed with the WISGEN software (version 7.1) of the University of Wisconsin Genetic Computer Group (Devereux *et al.*, 1984) and with the FASTA program (Pearson and Lipman, 1988).

Other recombinant DNA techniques

Standard procedures for recombinant DNA techniques were used essentially according to Sambrook *et al.* (1989). ^{32}P-Labelled DNA probes were obtained by random priming (Feinberg and Vogelstein, 1984) and end-labelled oligonucleotides by adding [γ-^{32}P]ATP with T4 polynucleotide kinase (Boehringer).

Amplification of tkt3 using the PCR method

Amplification of the third class of *tkt* genes from *Craterostigma* was achieved by PCR reactions with degenerate oligonucleotides. The specific sense primer 5′-TCGAATTC CCN GAR AAY CCN GGN G was derived from the amino acid sequence PENPGD (see Figure 1) with an *Eco*RI cloning site in addition. The antisense primer corresponded to the highly conserved sequence 470–475 of Tkt 7: 5′-ACGAATTC Y/GAT NGA RTC RTG NGT C. The expected size of the amplified fragment was 360 bp. Purified genomic DNA (20 ng) was subjected to 35 cycles of amplification using the following protocol: 4 µM each primer, 100 µM dNTPs, 10 mM Tris–HCl, pH 9.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100 and 2.5 units Taq DNA Polymerase (Pharmacia); 30 s denaturation at 94°C, 30 s annealing at 60°C and 1 min extension at 72°C. The DNA fragment obtained was cloned into the pBlueScript SK vector. Screening of the cDNA library with this PCR-amplified fragment led to the isolation of a 1808 bp cDNA insert, called *tkt3*.

Protein analysis

Electrophoresis under denaturing conditions was performed as described by Laemmli (1970) in 12 or 7.5% (w/v) SDS–polyacrylamide gels using Dalton Mark VI or VII (Sigma, Deisenhofen, Germany) as molecular mass markers. Protein concentrations were determined according to Bradford (1976) using the BioRad kit (Munich, Germany).

Expression cloning in E.coli, preparation of transketolase antiserum and immunodetection

From the cDNA clone *tkt10* a 975 bp fragment (nucleotides 125–1099 inclusive) was isolated and ligated into the *Sma*I site of the expression vector pGEX-T2 (Smith and Johnson, 1988) to yield a translational fusion with glutathione S-transferase. The expression of the fusion protein was induced in *E.coli* by the addition of isopropyl-β-D-thiogalactoside to 0.4 mM. Purification of the fusion protein by preparative gel electrophoresis, immunization of rabbits and Western blot analysis was performed as described in Bartels *et al.* (1991). The transketolase antiserum was shown to cross-react with the denatured forms of the *tkt3*-, *tkt7*- and *tkt10*-encoded proteins.

Purification of transketolase

Untreated *Craterostigma* leaves were homogenized with ice-cold extraction buffer (4 ml/g fresh weight) containing 50 mM Tris–HCl, pH 7.5, 10% (v/v) glycerol, 10 mM $MgCl_2$, 0.1 mM ThDP, 1.5% (w/v) polyvinylpolypyrrolidone, 5 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride , 1 mM benzamidine and 1 mM benzamide. The slurry was filtered through Miracloth and centrifuged for 10 min at 10 000 *g* followed by a centrifugation at 25 000 *g* for 30 min. The supernatant was fractionated by ammonium sulfate precipitation; active transketolase was contained in the 50–70% (w/v) $(NH_4)_2SO_4$ saturated pellet. The precipitate was dissolved in 10 ml buffer A [50 mM Tris–HCl, pH 7.5, 10% (v/v) glycerol, 10 mM $MgCl_2$] and loaded onto a DEAE–cellulose column (20×2.3 cm) (DE-52; Whatman) equilibrated with buffer A. The column was eluted using a linear gradient of from 0 to 0.5 M NaCl in buffer A (10 packed column volumes). Combined active transketolase fractions were concentrated in an Amicon flow cell with a membrane which selectively removed proteins below a molecular weight of 30 000; during this procedure the buffer was exchanged for buffer B (20 mM Tris–HCl, pH 8.15, 10 mM $MgCl_2$, 10% glycerol). The enzyme concentrate was applied to a FPLC Mono-Q HR 10/10 column (Pharmacia). The column was developed using a linear gradient of from 0 to 0.5 M NaCl in buffer B, using more than 20 column volumes. All steps except for the FPLC chromatography were carried out at 4°C.

Transketolase activity assay

Transketolase activity was assayed spectrophotometrically by measuring the decrease in the concentration of NADH at 340 nm. This assay monitors the generation of glyceraldehyde-3-phosphate from xylulose-

5-phosphate and ribose-5-phosphate, where the enzymes triose phosphate isomerase (TIM) and glycerol-3-phosphate dehydrogenase (GAPDH) link in a coupled enzymatic reaction. For the assays, appropriate amounts of plant extracts were incubated at 25°C in a final volume of 500 µl containing 50 mM Tris−HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mM ThDP, 0.2 mM NADH, 16 units/ml GAPDH, 50 units/ml TIM, 2 mM ribose-5-phosphate, 1 mM xylulose-5-phosphate.

Staining for transketolase activity in native gels

Proteins were separated in a discontinuous polyacrylamide slab gel using a 7.5% separating gel and a 4% stacking gel under native conditions at 4°C. The electrophoresis buffer systems were as given by Laemmli (1970), except that the gels contained 10% (v/v) glycerol and SDS was omitted. After the separation the proteins were stained *in situ* for transketolase activity using a modified procedure according to Feierabend and Gringel (1983). As in the enzymatic assay (see above), the detection of transketolase activity relies on the production of glyceraldehyde-3-phosphate, which is metabolized by GAPDH, generating NADH. NADH then reduces 2,2′-di-*p*-nitrophenyl-5′5′-diphenyl-3′3′-[3,3′-dimethoxy-4,4′ diphenylene]-ditetrazoliumchloride (NBT; Sigma). This reaction is mediated by phenazine methosulfate (PMS; Sigma); the reduced tetrazolium is a coloured, insoluble formazon. For this reaction the gel is incubated in a staining solution containing 50 mM Tris−HCl, pH 7.5, 0.7 mg/ml NAD, 2 mM $MgCl_2$, 10 mM sodium arsenate, 1 mM ThDP, 16 units/ml GAPDH, 0.4 mg/ml NBT, 23 µg/ml PMS, 1 mM xylulose-5-phosphate and 1 mM ribose-5-phosphate. The gel was incubated at 37°C in the dark until the blue colour developed (between 10 and 30 min).

Protein sequencing

Internal amino acid sequences were generated according to Eckerskorn and Lottspeich (1989). The Mono-Q protein fractions I and II were electrophoresed by 7.5% SDS−PAGE and bands stained with Coomassie blue (Serva). The transketolase-containing band (estimated 10–20 µg) was cut out, washed overnight with distilled water, dried slightly using a Speed Vac and incubated overnight at 37°C with cleavage buffer (25 mM Tris−HCl, pH 8.5, 1 mM EDTA, 1–2 µg endoprotease LysC; Boehringer, Tutzing, Germany). The resulting peptides were eluted for 4 h at 37°C with 60% acetonitrile containing 0.1% trifluoroacetic acid and separated by reversed phase HPLC. Eluting column, Merck Superspher 60RP select B; solvent A, 0.1% trifluoric acid in water; solvent B, 0.1% trifluoroacetic acid in an acetonitrile gradient of from 0 to 60% B over 60 min; flow rate 300 µl/min; detection wavelength 206 nm. Peptides were sequenced in a pulsed liquid phase sequencer 477A equipped with an on line PTH analyser 120A (both Applied Biosystems, Foster City, CA), according to the manufacturer's instructions.

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