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Plants contain two distinct classes of functional tryptophan synthase beta proteins

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ABSTRACT

Tryptophan synthase β-subunits (TSBs) catalyze the last step in tryptophan biosynthesis, i.e. the condensation of indole and serine yielding tryptophan. In microorganisms two subfamilies of TSBs (here designated as type 1 and type 2) are known, which are only distantly related. Surprisingly, in all genomes of multicellular plants analyzed genes encoding both types are present. While type 1 enzymes are well established as components of tryptophan synthase complexes, type 2 enzymes in plants have not yet been characterized. Tissue specific expression of the *TSB* genes from *Arabidopsis thaliana* was analyzed. While *AtTSB*1 is the predominantly expressed isoform in vegetative tissues, *AtTSB*1 and *AtTSBtype*2 reach similar transcript levels in seeds. AtTSBtype2 protein was expressed in *Escherichia coli* and purified. It converted indole and serine to tryptophan with a strikingly low K_m-value for indole of ca. 74 nM. *Attsbtype*2 T-DNA insertion mutants showed no obvious deviation from the wild type phenotype, indicating that AtTSBtype2 function is not essential under standard growth conditions. As example for a monocot enzyme, maize *TSB type* 2 was analyzed and found to be transcribed in various tissues. ZmTSBtype2 was also catalytically active and here a K_m-value for indole of ca. 7 μM was determined. These data indicate that TSB type 2 enzymes generally are functionally expressed in plants. Their potential biological role is discussed.

1. Introduction

The penultimate step of tryptophan biosynthesis is the cleavage of indole-3-glycerol phosphate to glyceraldehyde-3-phosphate and indole – the tryptophan synthase alpha (TSA) reaction. Indole then condenses with serine yielding tryptophan – the tryptophan synthase beta (TSB) reaction. In enterobacteria, such as *Escherichia coli* and *Salmonella typhimurium*, the two reactions are tightly coupled in a heterotetrameric tryptophan synthase complex, which allows efficient channelling of intermediary indole from the active site in TSA to the catalytic centre in TSB. In fungi tryptophan synthases occur as TSA–TSB fusion proteins, which also provides metabolic channelling of indole (for review, see Miles, 2001).

In plants tryptophan, in addition to its role as a protein component, is the precursor of the phytohormone indole-3-acetic acid (IAA), and a large number of defence compounds, such as indole alkaloids and indole glucosinolates (Kriechbaumer and Glawischnig, 2005). Consistent with this diversified function, plants typically contain two or more isoforms of TSA and TSB. Tryptophan synthase complexes are formed, which contain TSB subunits homologous to the TSBs of enterobacteria (Kriechbaumer et al., 2008; Radwanski et al., 1995), here referred to as type 1 TSBs. In maize, two highly homologous *TSB type* 1 genes are known, which are essential for tryptophan biosynthesis (Wright et al., 1992). *Arabidopsis thaliana* has three *TSB type* 1 genes: *AtTSB*1 (At5g54810), *AtTSB*2 (At4g27070) and *AtTSB*3 (At5g28237). Importance of AtTSB1 for tryptophan biosynthesis has been demonstrated based on mutant analysis (Last et al., 1991).

Recently an alternative type of TSBs (TrpB2) has been characterized in both Bacteria and Archeae (Hettwer and Sterner, 2002; Leopoldseder et al., 2006). To avoid ambiguity with the established nomenclature for maize and Arabidopsis, for TrpB2 homologs we here refer to TSB type 2. These type 2 beta subunits are only distantly related to the "classical" TrpB1 (type 1) enzymes and have been discussed to be the more ancient variants, from which type 1 enzymes have evolved during early prokaryote evolution (Merkl, 2007). The TSB type 2 enzymes from *Thermothoga maritima* and *Sulfolobus solfataricus* were analyzed biochemically. They were present as dimers and did not form a stable tryptophan synthase complex with the corresponding TrpA (TSA) protein (Hettwer and Sterner, 2002; Leopoldseder et al., 2006). Characteristic for both TrpB2 proteins was a low K_m-value for indole. For *T. maritima* it was proposed that the biological role of TSB type 2 is to prevent highly hydrophobic indole, which has escaped the tryptophan synthase complex, from travelling through the membrane and to channel it back into metabolism (Hettwer and Sterner, 2002).

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Surprisingly, a gene encoding a TSB type 2 homolog (*AtTSBtype2*; At5g38530) is also found in the genome of *A. thaliana*. It is more closely related to *T. maritima* TrpB2 or *S. solfataricus* TrpB2 than to the other TSBs in Arabidopsis. In this work we surveyed plant genomes for *TSB type* 2 genes and functionally analyzed the Arabidopsis and maize orthologs.

2. Results

2.1. TSB type 2 is widespread in plants

The large set of sequenced microbial genomes, allowed a comprehensive survey of TSB genes of type 1 and type 2. Among Bacteria and Archeae, phylogenetic subgroups, which contain either of the two or both types were identified (Merkl, 2007). We have now analyzed the genomes of the following plant species for TSB genes: *A. thaliana*, *Populus trichocarpa* (poplar), *Vitis vinifera* (grapevine), *Zea mays* (maize), *Sorghum bicolor*, *Oryza sativa* (rice), the lycophyte *Selaginella moellendorffii*, and the moss *Physcomitrella patens*. All these plant genomes analyzed contain at least one "classical" type 1 *TSB* gene. In dicots these type 1 genes cluster in two subgroups, an *AtTSB*1 group related to the monocot type 1 genes, and an *AtTSB*3 group (Fig. 1). In addition, in each genome there is one type 2 TSB gene present. These type 2 genes show a much higher degree of homology to the type 2 genes from Bacteria and Archeae (up to 70% identity on amino acid level) than to other plant *TSB* genes (e.g. AtTSB1/AtTSBtype2: 29% amino acid identity) (Fig. 1). The accession numbers of identified TSB genes, classified as type 1 or type 2 are available in Supplementary Table 1.

While in the genomes of the red alga *Cyanidioschyzon merolae* and the green algae *Ostreococcus lucimarinus* and *Chlamydomonas reinhardtii* only one *TSB type* 1 gene was detected, the green alga *Chlorella sp.* NC64A contains both type 1 and type 2 genes, each showing homology to corresponding genes from the cyanobacterium *Cyanothece sp.* PCC 7822 (Fig. 1). This indicates that both TSB types have been retained since the origin of plants, with possible loss of the type 2 orthologs in some lineages of algae.

To establish a potential biological role for plant TSB type 2, which so far have not been investigated, we have functionally analyzed the isoform from *A. thaliana*.

2.2. Expression pattern of A. thaliana TSB type 2

Publicly available array data (AtGenExpress) were analyzed for expression of *AtTSBtype*2 in relation to *AtTSB*1 and *AtTSB*2. In most tissues *AtTSBtype*2 expression was low in relation to *AtTSB*1. In contrast, *AtTSBtype*2 transcript levels strongly increased during seed development. *AtTSB*3 was not included in the Affimetrix array. To get a complete overview on *TSB* expression in *A. thaliana*, quantitative RT-PCR was performed in different tissues. In all tissues except seeds, *AtTSB*1 was expressed at much higher level in comparison to any of the other three *TSB* genes (Fig. 2). This observation is in accordance with a morphological phenotype of the *TSB*1 mutant *trp*2 (Last et al., 1991). In Arabidopsis, tryptophan biosynthesis is strongly induced upon pathogen infection, providing tryptophan as precursor for camalexin (for review see Glawischnig, 2007). Response to a Nep-1-like protein from *Phythium aphanidermatum* (PaNie), which triggers a multitude of pathogen defence reactions (Qutob et al., 2006) was assayed in plants expressing PaNie under control of an ethanol inducible promoter (Alc:PaNie) (Rauhut et al., 2009). The three type 1 isoforms were strongly induced by expression of PaNie toxin triggered by spraying Alc:PaNie plants with ethanol. This was not observed for *AtTSBtype*2 (Fig. 2A). In contrast to other tissues analyzed, *AtTSBtype*2 transcript levels in seeds were in the same range as *AtTSB*1 and *AtTSB*2 levels (Fig. 2B).

2.3. AtTSBtype2 insertion mutants are fully viable

Five putative T-DNA-insertion lines were analyzed for insertion in *AtTSBtype*2. Only SALK_011904 was confirmed containing a

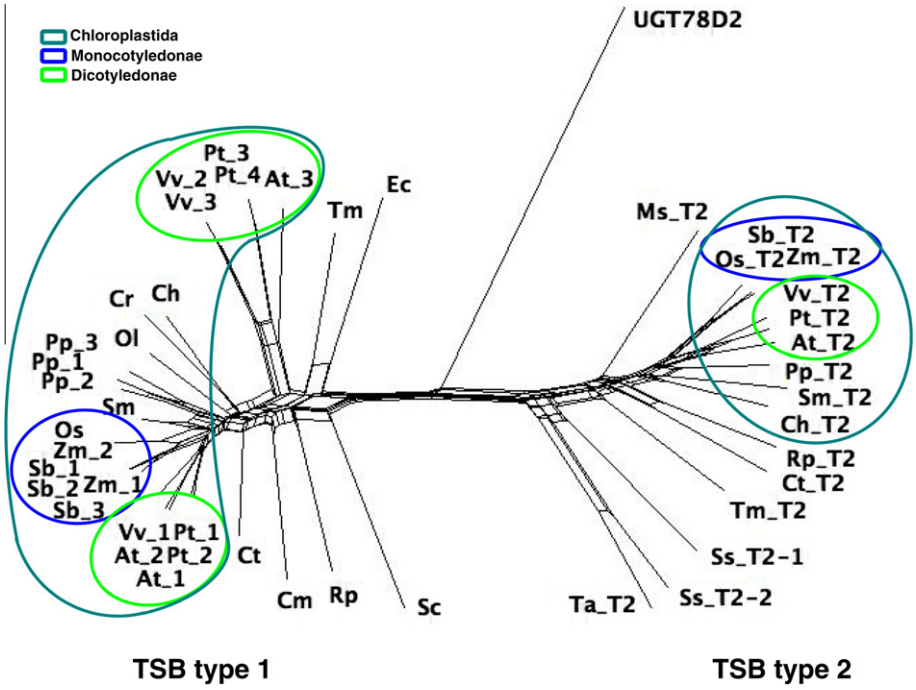

Fig. 1. Phylogenetic reconstruction of TSB protein sequences. For plants with full or extensive genome sequence information available and for selected microorganisms all *TSB* genes are indicated. All multicellular plants analyzed contained one "type 2" and at least one "type 1" gene. Phylogenetic reconstruction of the inferred amino-acid sequence was accomplished by SplitsTree (Huson, 1998). Type 1 genes: Acronym_isoform; type 2 genes Acronym_T2. Definitions of organismal acronyms and accession numbers are found in Supplementary Table 1. UGT78D2 (At5g17050) was used as outgroup.

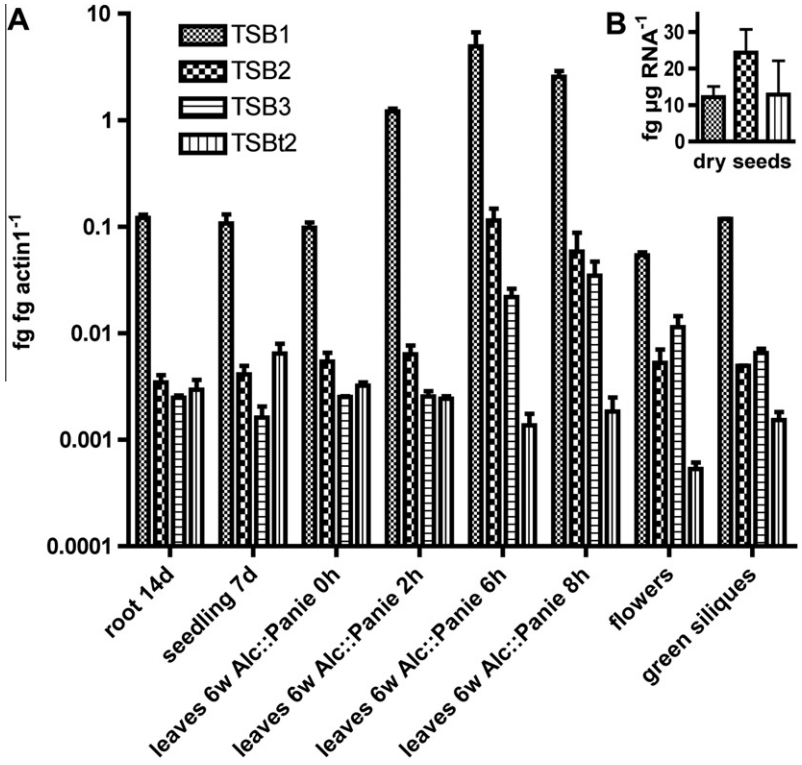

Fig. 2. Expression analysis of the four Arabidopsis *TSB* genes. (A) Transcript levels in different tissues of Col-0 WT or in rosette leaves of Col-0 plants expressing the Nep1-like protein PaNie under control of an alcohol inducible promoter 0, 2, 6 and 8 h after ethanol induction. *TSB*1 was predominant in all tissues. *TSB*1, *TSB*2, and *TSB*3 were strongly induced by PaNie expression. *Actin*1 was used for normalisation. (B) *TSB* expression in dry seeds. No reproducible detection of *TSB*3 and *Actin*1 reference gene could be achieved. Hence, here the data are normalized with respect to total RNA, $n = 3$.

T-DNA insertion in an exon. In addition, *AtTSBtype*2 was expressed under control of the 35S promoter. Three independent lines, each expressing *AtTSBtype*2 ca. 5–8-fold with respect to wild type, were subsequently used as biological replicates in phenotype analysis. No alteration in plant morphology was observed for T-DNA insertion lines in comparison to the wild type (data not shown). Specifically, seedling root growth was not significantly changed in the knockout mutant in comparison to the wild type (Supplementary Fig. 1).

2.4. TSB type 2 from A. thaliana shows very strong binding of indole

To test whether AtTSBtype2 protein has TSB activity, it was expressed as His-Tag fusion in *E. coli* BL21 (DE3) and purified. AtTSBtype2 readily converted indole and serine to tryptophan, which was quantified by fluorescence detection after HPLC separation. No product was observed in boiled control, or if indole or serine was missing from the reaction mixture. The kinetic properties of AtTSBtype2 were determined (Fig. 3). Strikingly, an extremely low K_m-value for indole of 74 ± 12 nM was detected. For serine a K_m-value of 35 ± 5 mM was determined. A k_{cat}-value of app. 0.01 s^{-1} (0.0070 s^{-1} (Fig. 3A)/0.0155 s^{-1} (Fig. 3B)) was determined, which is low in comparison to microbial TSB type 2 proteins (*T. maritima* TrpB2: 0.44 s^{-1}, *S. solfataricus* TrpB2i: 0.20 s^{-1}, TrpB2i: 0.032 s^{-1}), (Hettwer and Sterner, 2002; Leopoldseder et al., 2006).

Serine deaminase activity, which was proposed for TSB related proteins (Leopoldseder et al., 2006; Xie et al., 2002), was not observed.

The majority of freshly purified AtTSBtype2 protein separated by size exclusion chromatography (Kriechbaumer et al., 2008) eluted at ca. 90 kD (Supplementary Fig. 2). This is consistent with formation of homodimers, and similar observations have been reported for microbial TSB type 2 orthologs (Hettwer and Sterner, 2002; Leopoldseder et al., 2006).

2.5. Functional TSB Type 2 is expressed in maize

TSB type 2 genes are widespread, possibly universal, in the multicellular plants. Maize was chosen as a monocot species for functional analysis, as here TSA homologs and TSB type 1 enzymes have been previously analyzed in detail (Frey et al., 1997; Frey et al., 2000; Kriechbaumer et al., 2008; Wright et al., 1992). Based on *Z. mays* PCO109765 mRNA sequence, a full length *ZmTSBtype*2 cDNA clone was isolated from a λ-phage seedling cDNA library. The expression pattern of the maize *TSB* genes was analyzed by quantitative RT-PCR. Low but clearly detectable expression of *ZmTSBtype*2 was observed in all tissues analyzed (Supplementary Fig. 3) with no clear preference to developing kernels.

ZmTSBtype2 was expressed in *E. coli*, purified, and its kinetic parameters were determined (Fig. 3C and D). ZmTSBtype2 showed TSB activity, with an apparent K_m-value for serine of ca. 6.2 mM and for indole of ca. 6.8 µM. In comparison to AtTSBtype2 the strong affinity of ZmTSBtype2 towards indole was less pronounced. Again, a striking difference between the K_m-values for the two substrates was observed, which however was three orders of magnitude less in comparison to AtTSBtype2. A k_{cat}-value of app. 0.02 s^{-1} (0.010 s^{-1} (Fig. 3C)/0.022 s^{-1} (Fig. 3D)) was determined, which is one order of magnitude lower in comparison to ZmTSB1 (0.29 s^{-1}, Kriechbaumer et al., 2008).

3. Discussion

Tryptophan synthase beta genes have diverged into two distinct subclasses, here referred to as “type 1” and “type 2”, early

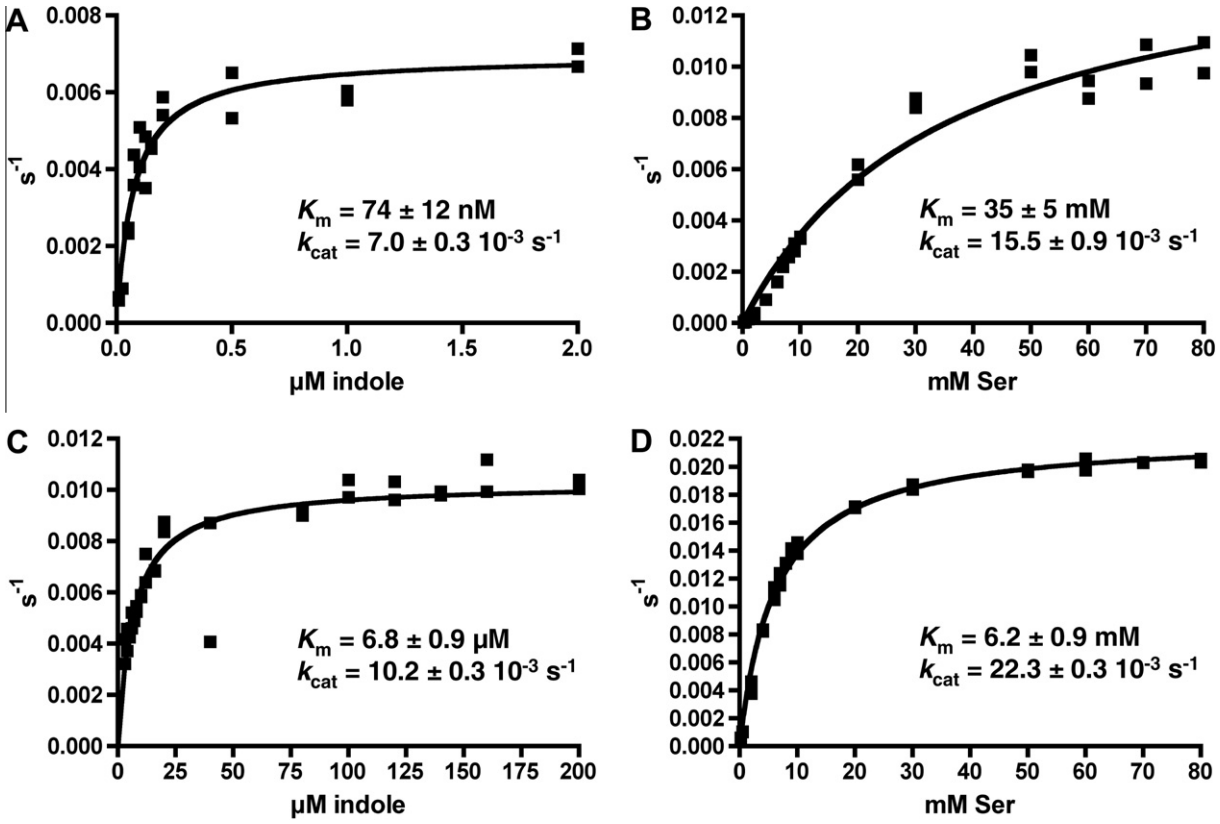

Fig. 3. Kinetic analysis of TSB activity. Tryptophan formation by purified AtTSBtype2 (A, B) or ZmTSBtype2 (C, D) was quantified applying varying concentrations of indole (A, C) or serine (B, D). A strikingly low K_m-value of ca. 74 nM was determined for AtTSBtype2 and the substrate indole.

in evolution of microorganisms (Merkl, 2007). Surprisingly the genomes of all vascular plants for which a full or largely complete sequence was available, the moss *P. patens*, and the green alga *Chlorella sp.* NC64A contain members of both types. This demonstrates that both types have been retained since the origin of plants and are widespread, if not universally found in higher plants. Therefore it is likely that type 2 enzymes have a unique function that was positively selected during plant evolution.

To address a potential biological role for TSB type 2 we have analyzed the expression pattern of the genes from maize and *A. thaliana* and characterized the corresponding enzymatic activities. In both the monocot and the dicot species the genes were expressed and the corresponding proteins were catalytically active. In particular the Arabidopsis enzyme exhibited a strikingly low K_m-value for indole. Based on their enzymatic characteristics, two different hypothetical functions for plant TSB type 2 proteins can be proposed:

(i) Biosynthesis of a metabolite analogous to tryptophan: Similar to TrpB2 proteins from microorganisms (Leopoldseder et al., 2006), the K_m-value for serine of TSB type 2 from Arabidopsis or maize was in the milimolar range. This contrasts with the characteristics of type 1 enzymes, e.g. ZmTSB1 has a K_m-value for indole of ca. 24 µM (Kriechbaumer et al., 2008). Therefore the question can be addressed, whether serine is a substrate and tryptophan indeed a product of TSB type 2 reaction *in vivo*. As a candidate reaction, we have tested AtTSBtype2 catalyzed condensation of glyoxylic acid and indole, which are the reactants in the chemical synthesis of indole-3-acetic acid (IAA) (Johnson and Crosby, 1973). No product formation was observed (data not shown), leaving it open, whether AtTSBtype2 catalyzes the reaction of indole with a molecule other than serine.

(ii) Efficient removal of free indole: For *T. maritima* it has been proposed that TrpB2 prevents metabolic loss of free indole by converting it to tryptophan (Hettwer and Sterner, 2002). Plants form tryptophan synthase complexes (Kriechbaumer et al., 2008), but these complexes are possibly leaky. In addition, functional TSA monomers (indole-3-glycerol phosphate lyases), such as maize Bx1 (Frey et al., 1997) and IGL (Frey et al., 2000) are optimized for the synthesis of free indole. Recent data suggest that such formation of free indole is widespread in plants (Schullehner et al., 2008). Indole is a known extracellular signal in bacteria (Lee et al., 2007; Wang et al., 2001). In maize, indole is synthesized by IGL as a signal for tritrophic interaction (Frey et al., 2000). Possibly, plants also use indole, synthesized by functional indole-3-glycerol phosphate lyases, as signalling compound for cellular regulation (Schullehner et al., 2008). As with all signalling processes, it is crucial that the initial signal is removable. TSB type 2 could possibly function in elimination of the transient indole signal. So far it is unclear, whether indole is a signalling component e.g. in Arabidopsis. We have surveyed publicly available transcriptomics data (AtGenExpress) for expression pattern of *AtTSBtype2*. Specific characteristic responses to phytohormones, biotic and abiotic stress signals, or co-regulation with *AtTSA* (At3g54640) or *AtTSA2* (At4g02610), which could be involved in indole formation, were not observed. Accordingly, *ZmTSBtype2* transcript levels (Supplementary Table 2)

did not correlate with *Bx*1 or *IGL* expression (Kriechbaumer et al., 2008). However, a regulatory circuit could also be functional with TSB type 2 being constitutively expressed at low levels allowing constant removal of free indole.

4. Conclusion

Tryptophan synthase beta type 2 enzymes are functionally expressed in both Arabidopsis and maize. They convert indole and serine to tryptophan with a strikingly low K_m-value for indole. Their biological function remains to be subject of speculation; as yet there is no clear evidence for a function of TSB type 2 in preventing loss of indole, signalling, or alternative biosynthetic pathways. No mutant phenotype for *Arabidopsis TSB type* 2 was yet observed, indicating TSB type 2 is not essential under optimal growth conditions. However, *TSB type* 2 genes are present in all genomes of multicellular plants completely sequenced so far and are phylogenetically related. This demonstrates that they have an important but yet undiscovered function for plant fitness.

5. Experimental

5.1. Sequence analysis

TSB protein sequences (Supplementary Table 1) were retrieved by homology search using Arabidopsis TSB1 (At5g54810) and TSB type 2 (At5g38530). Amino-acid sequences were aligned in ClustalW and a phylogenetic network was calculated with SplitsTree (Huson, 1998) excluding parsimony–uninformative sites, using UGT78D2 (At5g17050) as outgroup.

5.2. Plant material and growth conditions

Arabidopsis plants were grown in soil mixed with sand (3:1) in a growth chamber at 12 h light, 21 °C, 80–100 μmol of photons per square meter per second and 40% relative humidity.

SALK line 011904 was confirmed as T-DNA insertion mutant in the 1st exon of *AtTSBtype*2. SALK line 124293 was shown to carry a T-DNA insertion in the 4th intron, SALK lines 082810 and 162268 and SAIL_46_G06 could not be confirmed as insertion mutants in *AtTSBtype*2. For the generation of plants expressing *AtTSBtype*2 under the control of the 35S promoter the coding sequence was cloned with the USER strategy into pCambia 330035Su (Nour-Eldin et al., 2006) after amplification with the following primers: GGCTTAAUATGGCCTCTCAATTGCTTTTACC, GGTTTAAUTTAAACAACATGAGGAACCTTGG. Three independent overexpression lines, confirmed by quantitative RT-PCR using leave tissue, were phenotypically analyzed in detail.

5.3. RNA extraction and quantitative RT-PCR

Total RNA was isolated from the Arabidopsis Col-0 or the maize line B73 and quantitative real time PCR was carried out using the LightCycler/Syb®-Green dye system (Roche, Mannheim, Germany). Transcript analysis in maize tissues was performed as described (Kriechbaumer et al., 2008). Primers are given in Supplementary Table 2.

5.4. Protein expression and characterisation

The coding sequence of AtTSBtype2, excluding a 53 N-terminal amino acid putative plastid targeting sequence, was amplified from Col-0 leaf cDNA with the following primers: CTAGCTTAAGACATAT GGCAGCTTTGAGA/TTATGGGGCCATGGATCCT TAAACAACA. Based on *Z. mays* PCO109765 mRNA sequence, ZmTSBtype2 was isolated from cDNA bank of *bx*1 seedlings treated with volicitin (Frey et al., 2000). The coding sequence, excluding a 45 N-terminal amino acid putative plastid targeting sequence, was amplified with the following primers:GAGAAGCTTGCATATGACCACTAGGGCC/CATGTGCAGT TGGAGCTCGAATTC TCAGACTTTG. PCR products were cloned into pET28a His-tag vector, re-sequenced, and the proteins were heterologously expressed and purified under native conditions by His-tag affinity purification via Ni–NTA agarose and protein concentrations were determined colorimetrically (Bio-Rad Protein Assay), according to the manufacturers' suggestions (Qiagen, Hilden, Germany; Bio-Rad, Munich, Germany).

TSB reaction was performed with 2 μg purified protein at 30 °C, in 80 mM potassium phosphate buffer, pH 8.2 containing the following substrates: 50 μM indole, 60 mM L-serine, 50 μM pyridoxal phosphate; concentration ranges were analyzed for determination of kinetic parameters. For test of glyoxylic acid turnover 10 mM or 50 mM final concentration and pH 7.6 were used. After 20 min the reaction was stopped by the addition of 1 vol MeOH. Tryptophan and IAA were quantified by HPLC (RP-column: LiChroCART 125–4, RP-18, 5 μm; Merck, West Point, PA) using diode array (PDA-100, Dionex, Idstein, Germany) and fluorescence detection (RF-10A_{XL}, Shimadzu, Duisburg, Germany; excitation: 285 nm, emission: 360 nm). The mobile phase was delivered with a flow rate of 1 ml min^{-1} with an initial mixture of 15% (v/v) MeOH in 0.3% (v/v) HCOOH followed by a 15 min linear gradient to 100% MeOH. For calibration, standard curves were generated using authentic standards covering the relevant concentration ranges. Based on the determined protein concentrations and its molecular weight, turnover was calculated as nmol tryptophan s^{-1} $nmol^{-1}$ TSB type 2. k_{cat}-Values were determined in two series of measurements, varying indole or serine concentrations, respectively. Enzyme preparations were analyzed for serine deaminase activity for 4 h at 37 °C in 0.1 M KP_i, pH 7.8, 40 mM Ser, 10 mM reduced glutathione, 0.2 mM pyridoxal phosphate, 80 mM NADH, 190 U lactate dehydrogenase (Tsai et al., 1978).

For the detection of TSB type 2 dimerisation *in vitro*, size exclusion chromatography was performed as described (Kriechbaumer et al., 2008) in the following buffer: 100 mM Tris, pH 8.0, 100 mM KCl.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2010.07.006.

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