

9-2014

The Trehalose Pathway in Maize: Conservation and Gene Regulation in Response to the Diurnal Cycle and Extended Darkness

Clémence Henry

University of Nebraska-Lincoln, chenry3@unl.edu

Samuel W. Bledsoe

University of Nebraska-Lincoln

Allison Siekman

University of Nebraska-Lincoln

Alec Kollman


University of Nebraska-Lincoln

Brian M. Waters

University of Nebraska-Lincoln, bwaters2@unl.edu

See next page for additional authors

Follow this and additional works at: <https://digitalcommons.unl.edu/agronomyfacpub>

 Part of the [Agriculture Commons](#), [Agronomy and Crop Sciences Commons](#), [Ecology and Evolutionary Biology Commons](#), [Food Chemistry Commons](#), [Genetics Commons](#), and the [Plant Biology Commons](#)

Henry, Clémence; Bledsoe, Samuel W.; Siekman, Allison; Kollman, Alec; Waters, Brian M.; Feil, Regina; Stitt, Mark; and Lagrimini, L. Mark, "The Trehalose Pathway in Maize: Conservation and Gene Regulation in Response to the Diurnal Cycle and Extended Darkness" (2014). *Agronomy & Horticulture -- Faculty Publications*. 729.
<https://digitalcommons.unl.edu/agronomyfacpub/729>

This Article is brought to you for free and open access by the Agronomy and Horticulture Department at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Agronomy & Horticulture -- Faculty Publications by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Authors

Clémence Henry, Samuel W. Bledsoe, Allison Siekman, Alec Kollman, Brian M. Waters, Regina Feil, Mark Stitt, and L. Mark Lagrimini

RESEARCH PAPER

The trehalose pathway in maize: conservation and gene regulation in response to the diurnal cycle and extended darkness

Clémence Henry¹, Samuel W. Bledsoe¹, Allison Siekman¹, Alec Kollman¹, Brian M. Waters¹, Regina Feil^{1,2}, Mark Stitt^{1,2} and L. Mark Lagrimini^{1,*}

¹ Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE 68583-0915, USA

² Max Planck Institut für Molekulare Pflanzenphysiologie, Potsdam (OT) Golm, Germany

* To whom correspondence should be addressed. E-mail: mlagrimini2@unl.edu

Received 15 May 2014; Revised 10 July 2014; Accepted 11 July 2014

Abstract

Energy resources in plants are managed in continuously changing environments, such as changes occurring during the day/night cycle. Shading is an environmental disruption that decreases photosynthesis, compromises energy status, and impacts on crop productivity. The trehalose pathway plays a central but not well-defined role in maintaining energy balance. Here, we characterized the maize trehalose pathway genes and deciphered the impacts of the diurnal cycle and disruption of the day/night cycle on trehalose pathway gene expression and sugar metabolism. The maize genome encodes 14 trehalose-6-phosphate synthase (TPS) genes, 11 trehalose-6-phosphate phosphatase (TPP) genes, and one trehalase gene. Transcript abundance of most of these genes was impacted by the day/night cycle and extended dark stress, as were sucrose, hexose sugars, starch, and trehalose-6-phosphate (T6P) levels. After extended darkness, T6P levels inversely followed class II TPS and sucrose non-fermenting-related protein kinase 1 (SnRK1) target gene expression. Most significantly, T6P no longer tracked sucrose levels after extended darkness. These results showed: (i) conservation of the trehalose pathway in maize; (ii) that sucrose, hexose, starch, T6P, and TPS/TPP transcripts respond to the diurnal cycle; and (iii) that extended darkness disrupts the correlation between T6P and sucrose/hexose pools and affects SnRK1 target gene expression. A model for the role of the trehalose pathway in sensing of sucrose and energy status in maize seedlings is proposed.

Key words: Maize, shade stress, trehalose-6-phosphate, trehalose gene family, diurnal cycle, quantitative RT-PCR.

Introduction

A central feature of plant metabolism is the photosynthetic conversion of light energy into stored chemical energy. Every 24 h, plants cycle from net energy production to net energy consumption. During the day, plants produce sucrose and reducing sugars used in the synthesis of amino acids, lipids, nucleic acids, and complex carbohydrates. As light energy wanes at dusk and throughout the night, the plant transitions from a net producer of sugars to a net consumer. During the night, the plant utilizes stored carbohydrates as a source of carbon skeletons and

chemical energy (Baena-González *et al.*, 2007; Stitt and Zeeman, 2012). In some plants, like *Arabidopsis*, the vast majority of the stored carbohydrate is in the form of starch (Gibon *et al.*, 2009; Sulpice *et al.*, 2010, 2014), but in other plants, sugars including hexoses and sucrose can play a role in maintaining energy balance throughout the transition between light and dark. Trehalose [α -D-glucopyranosyl-(1 \rightarrow 1)- α -D-glucopyranoside] is an important osmotic protectant in bacteria, fungi, and insects where it accumulates to high concentrations (Avonce *et al.*,

Abbreviations: G6P, glucose-6-phosphate; HXK, hexokinase; RT, reverse transcription; SE, standard error; T6P, trehalose-6-phosphate; TPP, trehalose-6-phosphate phosphatase; TPS, trehalose-6-phosphate synthase; TRE, trehalose; UDGP, uridine diphosphate glucose.

© The Author 2014. Published by Oxford University Press on behalf of the Society for Experimental Biology.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/3.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

2006). Most plants accumulate only trace amounts of trehalose and its intermediates, where it is unlikely to function as an osmoprotectant (Paul *et al.*, 2008). Rather, the role of the trehalose metabolic pathway and its intermediates is to sense and communicate energy status (Lunn, 2007; Lunn *et al.*, 2014). As examples, exogenously applied trehalose altered physiology and gene expression, such as induction of the AGPase gene in *Arabidopsis* (Wingler, 2002), and resulted in increased biomass yield and water-deficit stress tolerance (Rodríguez-Salazar *et al.*, 2009; Sciences and Zeid, 2009; Ali and Ashraf, 2011). The inflorescences of the *ramosa3* mutant of *Zea mays* have significantly reduced trehalose (Carillo *et al.*, 2013) and excessive branching (Satoh-Nagasawa *et al.*, 2006). An induced increase in trehalose-6-phosphate (T6P) inhibits starch degradation in *Arabidopsis*, and changes in T6P modulate the photoperiod and flowering patterns (Wahl *et al.*, 2013).

Plants have a conserved three-step metabolic pathway for the synthesis and degradation of trehalose. In the first step, trehalose-6-phosphate synthase (TPS) catalyses the condensation of glucose-6-phosphate (G6P) and uridine diphosphate glucose (UDPG) to form T6P. Trehalose-6-phosphate phosphatase (TPP) subsequently removes phosphate to form trehalose. Trehalase (TRE) then hydrolyses trehalose into two glucose residues. Plant TPS and TPP are encoded by multi-genic families, while the trehalase (TRE) gene is present in a single copy (Lunn, 2007). *Arabidopsis* and rice genomes each encode 11 TPS genes and, respectively, 13 and 10 TPP genes (Yang *et al.*, 2012). TPS genes are divided into two classes. Class I TPS genes are generally present in a single copy, and they usually encode catalytically active TPS enzymes that have both TPS and TPP domains, with inactive phosphatase boxes. Class II TPS genes have both TPS and TPP domains but lack residues in the TPS domain needed for interaction with the substrate. Most class II TPS genes have conserved phosphatase domains; however, they do not possess TPS or TPP activity (Vandesteene *et al.*, 2010). In rice, some class II TPS proteins interact to form high-molecular-weight complexes, and a regulatory role is suspected (Zang *et al.*, 2011). All plant TPP genes are composed of a unique TPP domain with conserved phosphatase domains, and all encode functional TPP enzymes in *Arabidopsis*. Since they have similar activity but differential expression patterns, TPP genes probably have a tissue-, stage-, and/or process-specific function (Vandesteene *et al.*, 2012).

The diurnal switch from energy production to energy consumption requires a global change in gene expression and metabolic networks. In concert with the internal clock, sugar levels are a key regulator of this switch. Sugar levels fluctuate during the diurnal cycle, and sugars and circadian rhythm have an approximately equal and interactive effect on gene expression (Bläsing *et al.*, 2005). In maize, at least 10% of transcripts display circadian expression patterns, with peak expression at dawn and/or dusk in preparation for the periodic change in environment (Khan *et al.*, 2010). Not surprisingly, many diurnally regulated transcripts encode proteins involved in photosynthesis, respiration, carbohydrate metabolism, and cell elongation (Harmer *et al.*, 2000). Understanding how this switch takes place is of fundamental importance to improve crop productivity.

Plants have complex sugar signalling networks to maintain energy status regardless of photosynthetic output or growth

rate (Sheen, 2010). Hexoses are sensed through hexokinase (HXK)-dependent and HXK-independent pathways (Sheen, 2010). Sucrose sensing is less well understood; however, a correlation between sucrose and T6P levels strongly suggests that one route may involve a T6P inhibitory effect on sucrose non-fermenting-related protein kinase 1 (SnRK1), a global integrator of energy balance (Polge and Thomas, 2007; Zhang *et al.*, 2009; Nunes *et al.*, 2013). When energy levels decrease due to starvation or stress, SnRK1 is activated and triggers induction or repression of ~1000 genes to switch from anabolism to catabolism, promoting survival in lieu of growth (Baena-González *et al.*, 2007). This effect on gene expression probably involves the basic region leucine zipper transcription factor 11 (*bZIP11*) (Delatte *et al.*, 2011; Ma *et al.*, 2011).

As a consequence of altered carbohydrate metabolism (Lunn, 2007), dramatic phenotypes of plants with altered expression of trehalose pathway genes include effects on flowering, embryogenesis, branching, plant stature, biomass, grain yield, and abiotic/biotic stress tolerance (Wingler, 2002; Lunn *et al.*, 2014). The role of the trehalose pathway is significant; however, the details remain unclear. Recent evidence points to T6P having a central role in sugar sensing (Paul, 2007; Zhang *et al.*, 2009; Ponnu *et al.*, 2011; Wingler *et al.*, 2012; Wahl *et al.*, 2013). Sucrose and T6P levels were correlated in *Arabidopsis* meristems (Wahl *et al.*, 2013) and seedlings recovering from starvation (Lunn *et al.*, 2006), and developing wheat grain showed a close correlation between sucrose, T6P, and SnRK1 levels (Martínez-Barajas *et al.*, 2011), suggesting that T6P can act as a signal to indicate sucrose levels (Lunn *et al.*, 2014). Recent work in *Arabidopsis* showed the ratio between T6P and sucrose to be tightly regulated and critical to maintaining homeostasis throughout the diurnal cycle and during periods of stress (Yadav *et al.*, 2014).

Most work describing T6P and trehalose in energy-sensing networks has used *Arabidopsis*, which is a reference species for dicots and for C₃ photosynthesis. Little is known about the trehalose pathway gene structure, regulation, or role in central metabolism in the C₄ monocot maize, although maize is a major world crop that impacts on human and animal nutrition, and is an alternative energy source. The biodiversity in maize and availability of 'omics' data will be synergistic tools to investigate the impact of this pathway on plant growth and development.

This study aimed to identify and classify maize TPS/TPP/TRE gene families, and to determine their response to fluctuations in sugar and energy levels throughout the day/night cycle, after extended darkness (48 h) to impose an energy deficit, and during recovery from this dark treatment. Additionally, we compared starch/sucrose/hexose/T6P levels with TPS/TPP gene expression during recovery from extended darkness. A model is presented to integrate these new data into a more general view of the role of this pathway in plant growth and its response to the environment.

Materials and methods

Plant growth, treatment and harvest

Inbred B73 maize (*Z. mays* L.) plants were used. Seeds were sterilized for 15 min with 15% bleach (v/v), rinsed thoroughly with sterile water,

stirred for 1 min in 70 % ethanol, rinsed again, and soaked for 5 min in sterile water. Seeds were then rolled in germinating paper (Anchor Paper) and germinated for 4 d in the presence of 1 mM CaSO_4 solution in a growth chamber (16 h day/8 h night, $220 \pm 30 \mu\text{mol m}^{-2} \text{s}^{-1}$, 24 °C, 50% relative humidity). Seedlings were planted in germinating trays containing potting mix (34% peat, 31% perlite, 31% vermiculite, 4% soil), grown under the same conditions as described previously and watered daily with nutrient solution (20-20-20; J. R. Peters). Thirteen-day-old juvenile plants were then placed under a control photoperiod (same as above) or shaded for 48 h (frame covered with a thick black fabric shading cloth, $75 \times 75 \times 45 \text{ cm}$; $0 \mu\text{mol m}^{-2} \text{s}^{-1}$), and the frame was then removed to permit recovery for an additional 48 h (Brouquisse *et al.*, 1998; Mutisya *et al.*, 2009). Leaf 3 (fully-expanded) was harvested every 8 h for 48 h starting at the end of the dark period (first time point: 6 a.m., end of the night) from plants randomly picked in the tray (Fig. 1). The centre one-third of the leaf (100–200 mg) was collected into pre-chilled microcentrifuge tubes, instantly frozen in liquid nitrogen, and stored at $-80 \text{ }^\circ\text{C}$ until use.

Gene identification and bioinformatic analysis

Genes were identified using a name search and BLAST with *Arabidopsis* sequences in the <http://www.maizesequence.org> and <http://bioinformatics.psb.ugent.be/plaza/databases>. Predicted protein sequences were then compared with those of rice, *Arabidopsis*,

and poplar (Supplementary Tables S1 and S2 at *JXB* online) using the following website to generate classification and phylogenetic relationships: <http://www.phylogeny.fr/> (alignment with MUSCLE, phylogeny with PhyML, and tree rendering with TreeDyn). Protein sequences were also analysed using <http://myhits.isb-sib.ch/> software with the following parameters (hamap, pat, prf, pre, pfam_fs, and pfam_Is) to identify conserved TPS and TPP protein domains.

Gene expression analysis

Frozen tissues were grounded to a fine powder using a Tissue Lyser II (Qiagen). RNA was extracted using a Trizol protocol as described by the provider with the addition of 1 μl of glycogen (Invitrogen) at the beginning. RNA samples were DNase treated using RQ1 RNase-free DNase (Promega) as recommended by the supplier and stored at $-80 \text{ }^\circ\text{C}$ until use. RNA quantity and quality were checked using a Nanodrop 8000 spectrophotometer (ThermoScientific) and electrophoresis on a 1.2% agarose gel, respectively.

Reverse transcription (RT) was performed on 1 μg of total RNA using a SuperScript III First-Strand Synthesis Supermix kit (Life Technology) with random hexamer primers. RT quality and absence of genomic DNA contamination was then checked by semi-quantitative PCR using 5 μl of cDNA at a 1:100 dilution in a final volume of 25 μl using GoTaq® DNA Polymerase (Promega). ZmEF1-1 α primers (forward: 5'-AGACTCACATCAACATTGTGGTCAT-3', reverse: 5'-GT TGT CAC CT TCAAACCAGAGATT-3') were designed around an intron. For real-time RT-PCR, 5 μl of cDNA at a 1:50 dilution was used for reactions with SsoAdvanced™ SYBR® Green Supermix (Bio-Rad) and 167 nM primers (Supplementary Table S2) in a final volume of 15 μl . Real-time amplification was performed in the LightCycler® 480 II (Roche) using the following program: 30 s at 95 °C; and 45 cycles of 5 s at 95 °C, 30 s at 60 °C, and 10 s at 72 °C. A melting-curve analysis was performed for 5 s at 95 °C, followed by 5 °C increments from 65 to 95 °C. For each time point and three biological replicates, quantitative PCR was performed three times. Six reference genes (Supplementary Table S2) were tested and three were selected using the Genorm software (Vandesompele *et al.*, 2002). Relative gene expression was then calculated using the formula of Hellemans *et al.* (2007). Primer efficiency was determined using the method described by (Pfaffl (2001).

Carbohydrate metabolite analysis

Frozen tissues (20–100 mg) were weighed and ground for 30–60 s while frozen using a Tissue Lyser II (Qiagen). Sugars (sucrose, fructose, and glucose) were then extracted following the method of Lunn *et al.* (2006) using lactose as an internal standard. Starch was extracted from the pellet generated during the extraction of soluble sugars, and quantified by analysis of glucose resulting from hydrolysis (Supplementary Methods S1 at *JXB* online). Samples were analysed with a high-pressure capillary ion chromatograph system (ICS-5000, PA-20 column; Thermo Scientific Dionex) using a 1 μl injection volume and 45 mM KOH eluent. Sugar peaks were identified in comparison with known sugars, and data were analysed using the formulae described in Supplementary Methods S2 at *JXB* online. The method of Lunn *et al.* (2006) using anion-exchange liquid chromatography, linked to tandem mass spectrometry, was used to quantify T6P.

Statistical methods

Pearson correlation coefficient matrices between transcripts, sugars, and transcript versus sugars were determined were computed using the stats package from R software version 3.0.1 (R Core Team, 2013, <http://www.r-project.org/>). The average of three biological replicates was used to perform tests. Heat maps were then generated in MS Excel using conditional formatting functions.

Student's *t*-test was used to compare differences between control and shaded condition for each time point. Analysis was performed using the following website: <http://www.physics.csbsju.edu/stats/t-test.html>. Significant differences with a value of $P < 0.05$ are indicated by an asterisk.

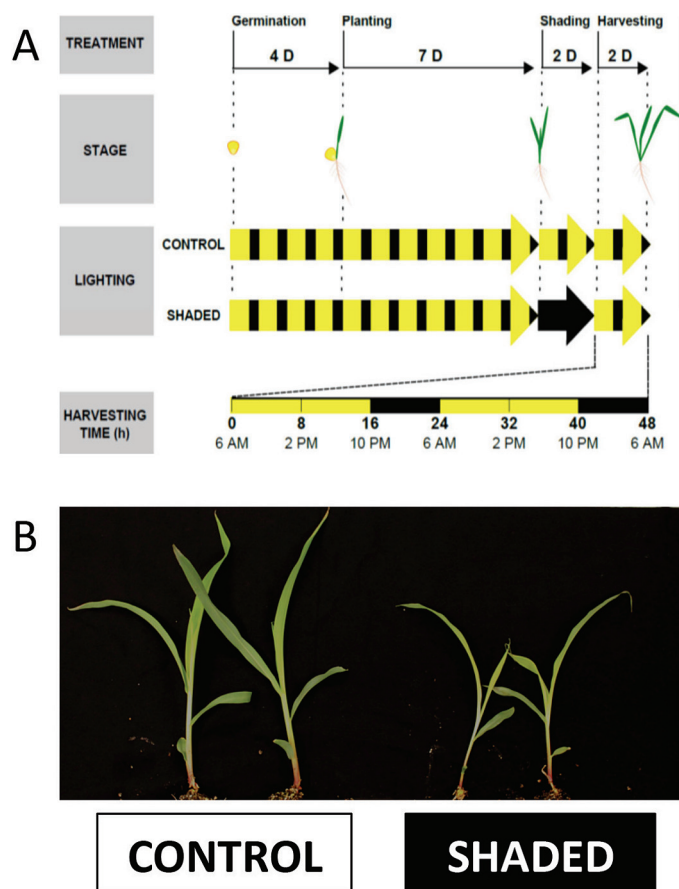


Fig. 1. Experimental design (A) and plant phenotype after 48 h of treatment (B). (A) B73 maize seeds were germinated for 4 d, planted, and cultivated under control diurnal cycles (16 h day (D)/8 h night (N), $220 \pm 30 \mu\text{mol m}^{-2} \text{s}^{-1}$, 24 °C, 50% relative humidity) for 7 d. The plants were then kept under the same conditions (control) or under total darkness (shaded) for 48 h. They were then returned to regular diurnal cycles for recovery. Sample harvesting was done every 8 h for 48 h, starting at the end of the dark period when plants were still under darkness. (B) Control and shaded plants grew at different rates as a result of the treatment.

Results

Classification of maize TPS/TPP genes

We identified 14, 11, and one genes predicted to encode for TPS, TPP, and TRE maize enzymes, respectively (gene accession numbers in Table S1) from maize genome databases (<http://www.maizesequence.org> and <http://bioinformatics.psb.ugent.be/plaza/>). The maize genome also encodes several genes with truncated TPS/TPP domains, named TPS-like or TPP-like. These are unlikely to be functional trehalose pathway enzymes based on domain analysis using the MyHits tool; therefore, we did not investigate them further.

As described by Yang et al. (2012), TPS genes were divided into two clades: clade B included all class I TPS genes while clade A included all class II TPS genes. Clades B and A subdivided into two and five subclades, respectively, corresponding to groups with common ancestors before the split between monocots and dicots. Class A was found in all dicots and subclade B2 was specific to *Arabidopsis*. Maize encoded two class I TPS genes (clade B) and 12 class II TPS genes (clade A), named according to their position in the phylogenetic tree (Fig. 2A). All maize TPS proteins included both a TPS and TPP domain. Among the class I TPS genes, clade B1 contained the functional TPS from rice and *Arabidopsis* and both maize TPS class I genes. *ZmTPSI.1.1* (previously

named *ZmTPSI*) encoded a functional TPS enzyme and had all conserved TPS motifs (Table 1, Supplementary Fig. S2 at JXB online) (Jiang et al., 2010). Structurally, *ZmTPSI.1.2* was a truncated version of *ZmTPSI.1.1* and was missing amino acids required for substrate binding. This gene is therefore unlikely to encode a functional TPS enzyme. Interestingly, all class I TPS proteins lacked the first phosphatase motif required for the catalytic activity, although they possessed a full TPP domain. Maize class II TPS genes were composed of subclades A2–A5 with *ZmTPSII.3.1*, -3.2, and -3.3; *ZmTPSII.4.2* and -4.3; *ZmTPSII.5.1* and -5.2; and *ZmTPSII.5.3* and -5.4, respectively. Maize class II TPS enzymes had a substitution of arginine with aspartic acid in the UDPG phosphate-binding pocket (Table 1). Most maize class II TPS displayed substitution of three to four amino acids in the UDPG- and G6P-binding sites, while class II TPS genes belonging to clade A5 showed a higher number of substitutions in the UDPG-binding site but had a highly conserved G6P-binding site.

The TPP family also had two clades that were divided into three and two subclades (Fig. 2B). Subclades A1 and B1 included genes from both monocots and dicots, while other subclades were specific to monocot species. The position of the maize TPP genes within subclades was used for their nomenclature. TPP genes mainly evolved through duplication

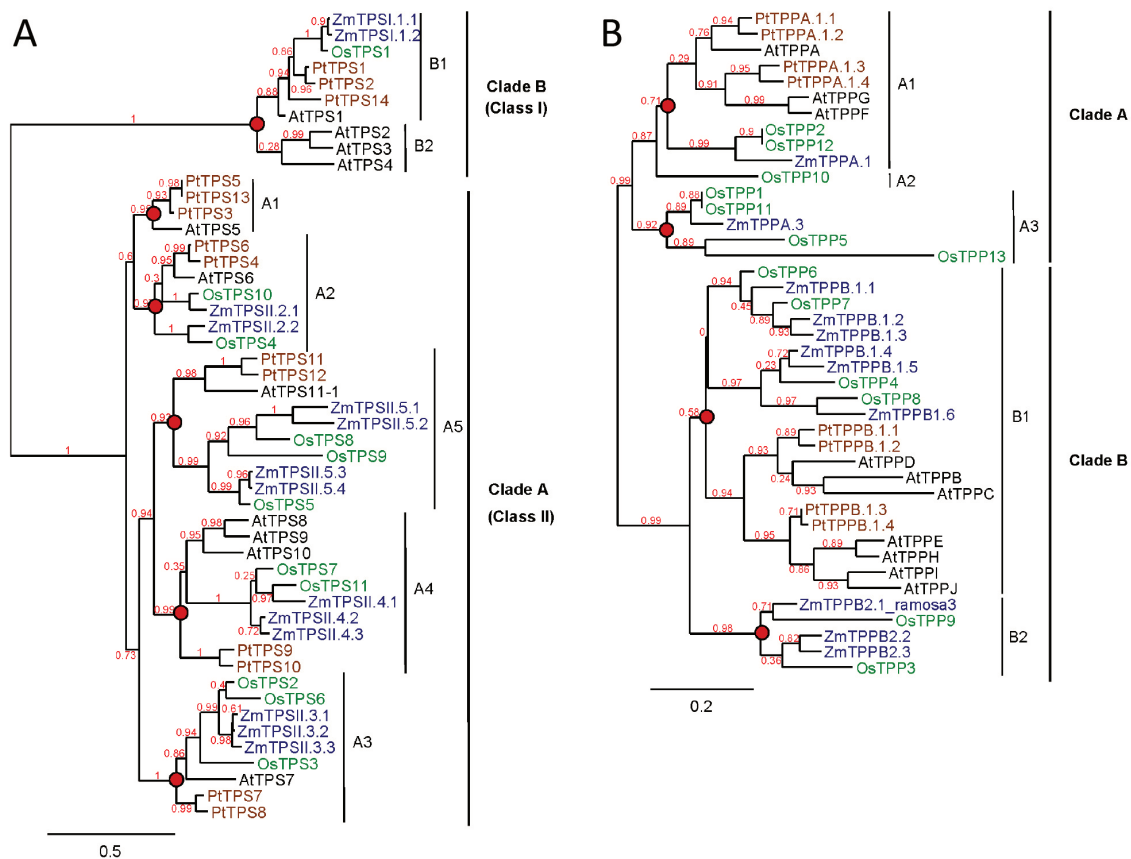


Fig. 2. Phylogenetic trees of TPS and TPP genes from *Z. mays*, *Arabidopsis thaliana*, *Oriza sativa*, and *Populus trichocarpa*. A phylogenetic tree was developed using predicted protein TPS (A) and TPP (B) sequences from maize (blue), *Arabidopsis* (black), rice (green), and poplar (brown) identified from genomic databases (<http://www.maizesequence.org> and <http://bioinformatics.psb.ugent.be/plaza/>). Common ancestors before the split between monocots and dicots are indicated by red circles. Clades and subclades are indicated and bootstrap values are shown in red. Bars, amino acid substitutions per site.

Table 1. Conserved amino acids required for TPS and TPP activity in TPS predicted proteins from *E. coli*, *Arabidopsis* and maize

Residues involved in substrate interaction of the TPS domain are designed by a letter associated with a number indicating their position (Gibson *et al.*, 2002; Vandesteene *et al.*, 2010). Deletions are represented by a X. Presence (+) or absence (-) of the three phosphatase motifs required for the activity in the TPP domain is also indicated (Avonce *et al.*, 2006; Lunn, 2007). Conservation of residues or motifs required for TPS and TPP activity is highlighted by shading.

Substrate / Motif	TPS CLASS I proteins										TPS CLASS II proteins									
	OsTPS1.1	ZmTPS1.1	ZmTPS1.2	ZmTPS1.2.1	ZmTPS1.2.2	ZmTPS1.3.1	ZmTPS1.3.2	ZmTPS1.3.3	ZmTPS1.4.1	ZmTPS1.4.2	ZmTPS1.4.3	ZmTPS1.5.1	ZmTPS1.5.2	ZmTPS1.5.3	ZmTPS1.5.4					
UDP-G																				
Uracil		L446	L469	L83	L432	L419	V454	V454	V454	I419	I417	I417	M515	L425	V543	V412				
Ribose		G120	G142	X	D94	D86	D106	D106	D106	N88	D86	D86	D179	D90	G210	G79				
		E476	E498	E113	E462	E449	E484	E484	E484	E449	E447	E447	I545	I455	I573	I442				
Phosphate		R369	R391	R6	D355	D342	D377	D377	D377	D342	D340	D340	D437	D348	D466	D335				
		K374	K396	K11	K360	K347	K382	K382	K382	K347	K345	K345	K442	N553	K471	K340				
		N471	N493	N108	N457	N444	N479	N479	N479	N444	N442	N442	N540	N450	N568	N437				
		L472	L494	L109	L458	L445	L480	L480	L480	L445	L443	L443	R541	R451	R569	R438				
		V473	V495	V110	I459	I446	T481	T481	T481	I446	V444	V444	T542	T452	I570	I439				
Glucose		H261	H283	X	H241	H228	H625	H265	H265	H265	H228	H228	H325	H236	H354	H223				
		H292	H314	X	H272	H259	H296	H296	H296	Q261	Q259	Q259	H356	Q267	H385	H254				
		D468	D490	D105	D454	D441	D476	D476	D476	D441	D439	D439	D537	D447	D565	D434				
		M470	M492	M107	M456	M443	M478	M478	M478	M443	M441	M441	L539	L449	L567	L436				
		N471	N493	N108	N457	N444	N479	N479	N479	N444	N442	N442	N540	N450	N568	N437				
G6P																				
Glucose		R100	R122	X	Q69	Q64	Q85	Q85	Q85	Q88	F67	F66	R157	R68	R188	R57				
		W185	W207	X	W166	W153	W173	W173	W173	W176	W155	W153	W245	W156	W277	W146				
		D237	D259	X	D217	D204	D241	D241	D241	D241	D206	D204	D301	D312	D330	D199				
		T262	T284	X	S242	S229	S266	S266	S266	S266	S231	S229	T326	S237	S355	S224				
Phosphate		R100	R122	X	Q69	Q64	Q85	Q85	Q85	Q88	F67	F66	R157	R68	R188	R57				
		Y176	Y198	X	Y17	Y144	Y164	Y164	Y164	Y167	Y146	Y144	Y236	Y147	Y268	Y137				
		H133	H239	H261	H219	H206	H243	H243	H243	H243	H208	H206	H303	H214	H332	H201				
		R407	R429	R44	R393	R380	R415	R415	R415	R415	R380	R378	R475	R385	R504	R373				
Phosphatase																				
Motif I	[-]	[-]	[-]	[-]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]				
Motif II	[-]	[+]	[+]	[-]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]				
Motif III	[-]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[+]	[-]	[-]	[+]	[+]	[+]	[+]				

events after the monocot/dicot split or even after speciation, in contrast to TPS genes. All maize TPP genes displayed three conserved motifs required for TPP activity: (i) DXDX(T/V) (L/V/I); (ii) (S/T)(GX) in an hydrophobic context; and (iii) K(X)16–30(G/S)(D/S)XXX(D/N) (Table 1, Fig. S2) (Avonce et al., 2006; Lunn, 2007).

Maize TPS/TPP and SnRK1 targets gene expression

Maize TPS/TPP gene expression was characterized throughout the regular diurnal cycle and during the recovery from 48h of extended darkness. In plants with regular diurnal

cycles, gene expression patterns were quite varied among TPS/TPP genes and putative SnRK1 targets (Fig. 3). Expression of the catalytically active *ZmTPSI.1.1*, *ZmbZIP11*, and *ZmTPPB.1.3* increased throughout the morning, peaking at 2 p.m., and then decreased in the late afternoon and night. Most class II genes and *ZmTPPA.1* had their highest transcript levels at the end of the night period and decreased throughout the day. Several SnRK1 target genes were selected as indicators of a possible SnRK1 activity. We looked at the expression of some targets shown to be upregulated (β Gal, AKIN β , and ARG10) or downregulated (MDH, bZIP11, and DPS) by SnRK1 in *Arabidopsis* (Supplementary Fig. S3D

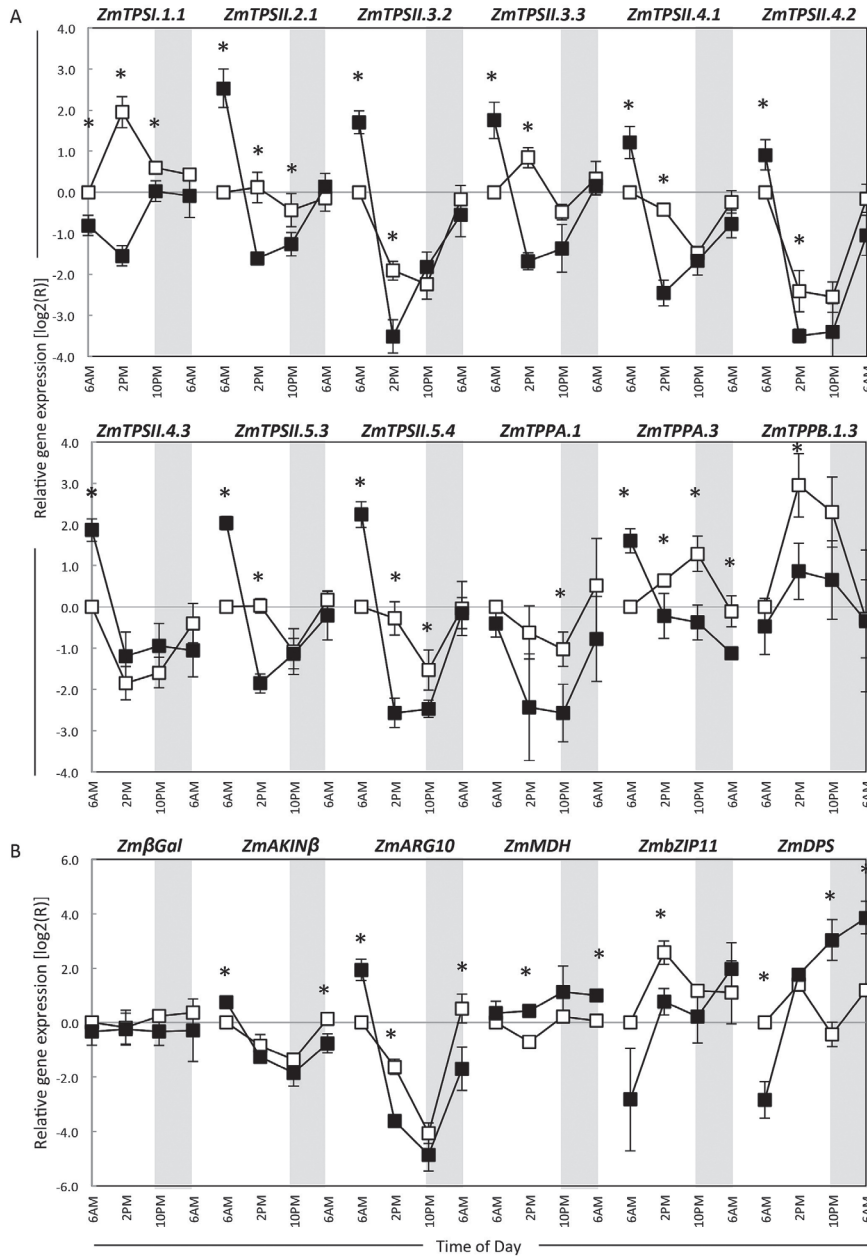


Fig. 3. Relative gene expression for selected maize TPS, TPP, and SnRK1 putative target genes in control and shaded seedlings. Leaf tissue were collected from the V3 stage control (16h day/8h night, open squares) and shaded (48h, filled squares) plants. Sampling was done every 8h for 24h starting at the end of the night or extended shading period (recovery phase). TPS and TPP transcript levels were specifically measured using quantitative RT-PCR. Transcript levels are expressed relatively to the first time point of control treatment. Expression was normalized to the geometric mean of stably expressed genes: *ZmPP2AA2-2* and *ZmCACS*, and transformed using a log₂ function. Data are presented as means±standard error (SE) of independent biological samples ($n=3$). Genes are grouped in three classes (A, B, and C) based on their response to regular circadian cycles (open squares).

and E at *JXB* online) (Baena-González *et al.*, 2007; Usadel *et al.*, 2008). As with the class II TPS genes, SnRK1 inducible transcripts were highest at the end of the night period and SnRK1-repressible transcripts were lowest. *ZmTPSII.2.1*, *ZmβGal*, and *ZmMDH* showed no significant change in transcript levels throughout the day/night period.

In plants recovering from extended darkness, all of the class II TPS genes had significantly higher transcript levels at the end of the dark period compared with control plants at the end of an 8 h night. Transcript levels fell between 8 and 16 h in the first light period after extended darkness (2 and 10 p.m.) and returned to normal levels by 24 h after shading ended. Dark stress resulted in reduced expression of *ZmTPSI.1.1* at a time it normally peaks during day/night cycle, and then returned to cycling similar to the control plants. A similar but attenuated pattern was observed for *ZmTPPA.3* and *ZmTPPB.1.3*. Among the putatively upregulated SnRK1 targets, two responded accordingly: *ZmAKINβ* and *ZmARG10* were both decreased during the day and increased at night in control plants (Fig. 3). These genes were induced by extended darkness and then repressed during the day during recovery, similarly to all class II TPS genes tested. Among the putatively downregulated targets, two, *ZmbZIP11* and *ZmDPS*, responded as expected: under extended darkness they were strongly repressed and then induced during recovery.

To determine if genes were regulated in a similar fashion, we determined their coefficient of correlation in control conditions and in dark-treated plants in the 24 h following the treatment (Table 2). In control conditions, expression of some class II TPS genes positively correlated to each other, while most of them negatively correlated with *ZmTPPA.3* expression. Most of class II TPS genes positively correlated with upregulated targets of SnRK1 (*ZmAKINβ* and *ZmARG10*). Other correlations were not as clear. In dark-treated plants, correlations between gene expression were much more obvious. All class II TPS transcripts were positively correlated to each other, to *ZmTPPA.1*, and to SnRK1 upregulated targets. In contrast, their expression was negatively correlated to *ZmTPPB.1.3* expression and negatively correlated with putative SnRK1 downregulated targets (*ZmbZIP11* and *ZmDPS*). Expression of *ZmTPSI.1.1* positively correlated with *ZmMDH* in dark-treated plants.

Soluble sugars and starch in cycling and dark-treated plants

Under regular diurnal cycling, concentrations of sucrose (Fig. 4A) and starch (Fig. 4D) were lowest in the morning (6 a.m.), rose slightly in the first 8 h of light, strongly between 8 and 16 h in the light, and decreased overnight. Starch levels were higher than sucrose at dusk, indicating that it was the major transient carbon store in maize. The delay in the onset of starch accumulation resembled that seen in *Arabidopsis* in long photoperiods (Sulpice *et al.*, 2014). Extended starvation stress affected starch and sucrose accumulation, but each in a different way (Fig. 4; filled squares). Unexpectedly, both were higher in leaf 3 at the end of a 48 h period of shading than at the end of the 8 h night, including

a 3-fold higher level of starch. This was in contrast to whole *Arabidopsis* rosettes, where starch and sucrose were very low after 48 h of darkness (Usadel *et al.*, 2008). During recovery, sucrose accumulated during the light period, but this increase occurred earlier, by at least 8 h (Fig. 4A). This response was attenuated on the second day, indicating a return to a regular diurnal pattern of regulation. During recovery from shading, starch showed a dramatically different response to that in an undisturbed light/dark cycling. Starch decreased during the first 8 h in the light and rose to a high level at dusk similar to that in control plants but remained high at the end of the night instead of being degraded (Fig. 4D). The decrease in starch during the first part of the light period coincided with an increase in sucrose levels (compare Fig 4A and D). Glucose and fructose had low levels and a less distinct diurnal pattern, and shading had a minor effect on their levels (Fig. 4B and C).

T6P response to diurnal cycling and recovery from extended darkness

T6P levels were measured using liquid chromatography coupled with tandem mass spectrometry (Fig. 5, Table 3). Under regular diurnal cycles, T6P levels were low in the morning, went up during the day to reach their maximum in the evening, and then decreased overnight. After 48 h of darkness, T6P levels were significantly lower than in control plants in the morning and at noon, and then rose to reach levels like those in the control in the evening. During the night, T6P levels decreased but less than in control plants, and again were lower than in controls at noon the on the second day of recovery, and then rose to levels like those in the control at dusk. Additionally, T6P levels were correlated with sugar and starch levels throughout the diurnal cycle but not after extended darkness (Table 3).

TPS/TPP gene expression versus carbohydrate and T6P levels

To help visualize the relationship between TPS/TPP gene expression and sugar levels, we determined the correlation between sugar and transcript levels over the first 24 h of treatment using a Pearson test (Table 4). Under control conditions, transcript levels of some class II TPS (*ZmTPSII.4.1*, -5.3, and -5.4) and SnRK1 upregulated (*ZmAKINβ* and *ZmARG10*) targets negatively correlated with T6P, sucrose, and fructose, and sometimes with glucose and starch. *ZmTPPA.1* behaved similarly and correlated negatively with T6P and fructose. *ZmTPSII.2.1* negatively correlated with sucrose, glucose, and starch, while *ZmTPSII.3.3* correlated negatively with glucose and starch only. Conversely, *ZmTPPA.3* expression correlated positively with T6P, sucrose, and fructose levels. In plants recovering from extended darkness, most class II TPS transcript levels tended to correlate negatively with sucrose and sometimes fructose levels. Conversely, *ZmTPPB.1.3* correlated with sucrose levels. Interestingly both *ZmTPSI.1.1* and *ZmMDH* (malate dehydrogenase) correlated strongly with T6P and starch levels.

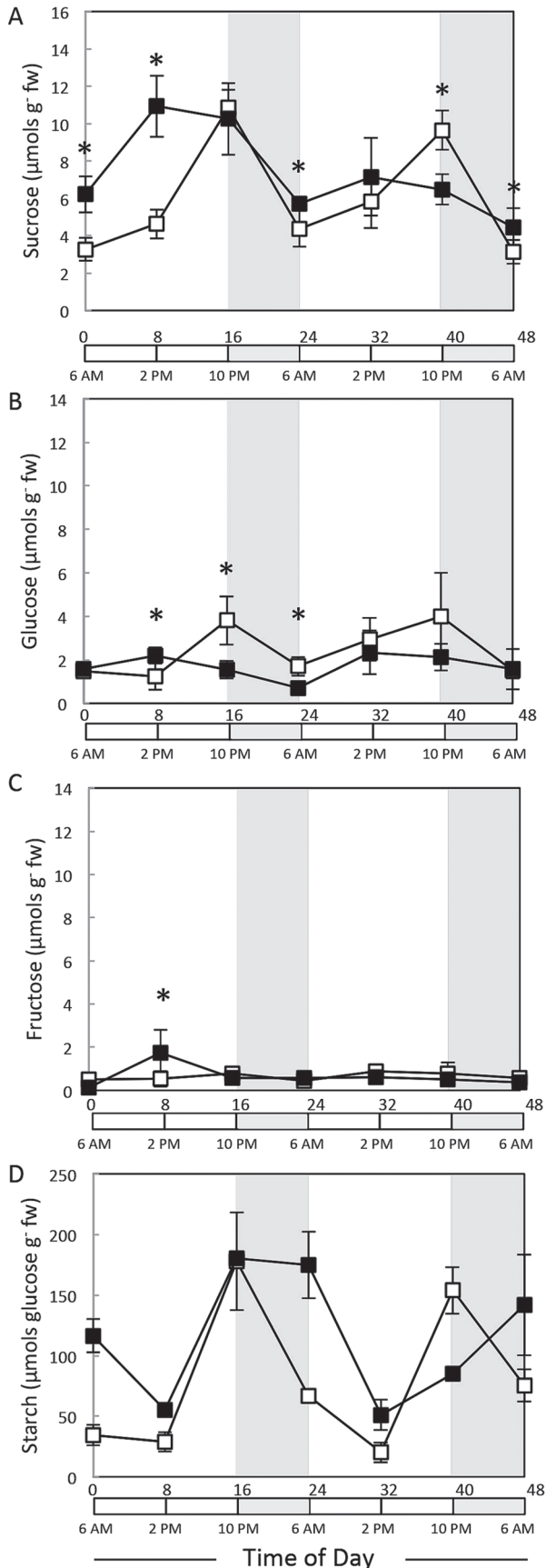


Fig. 4. Temporal levels of soluble sugars and starch in maize control or shaded seedlings. Starch and soluble sugars were extracted from the V3 stage control (16h day/8h night, open squares) and shaded (48h, filled squares) plants. Sampling was done every 8h for 48h starting at the end of

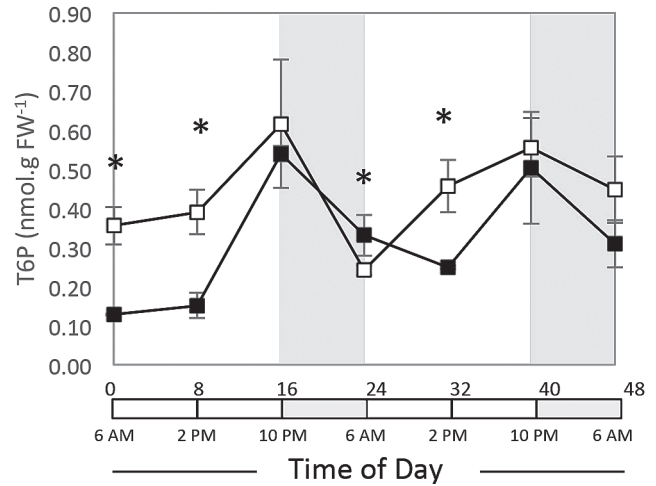


Fig. 5. Temporal levels of T6P in maize control or shaded seedlings. Sugars were extracted from the V3 stage control (16h day/8h night, open squares) and shaded (48h, filled squares) plants. Sampling was done every 8h for 48h starting at the end of the night or extended dark period (recovery phase). T6P levels were determined using reversed-phase liquid chromatography, linked to tandem mass spectrometry. Values are presented as means \pm SE of independent biological replicates ($n=3$).

Discussion

Crop productivity depends on the efficient conversion of solar energy into grain and biomass. Many of these efficiencies are realized through the carefully orchestrated metabolic switch between day and night. This large-scale metabolic switch is closely regulated by the circadian clock and through the sensing of intracellular sugar levels (Bläsing *et al.*, 2005). Over the last decade, the trehalose pathway and its intermediate precursor, T6P, have surfaced as key regulators of carbohydrate metabolism, growth, and development in several plant species (reviewed by Paul *et al.*, 2008; Schluempmann *et al.*, 2011; Lunn *et al.*, 2014). Much of the research on the trehalose pathway has used the model C_3 plant *Arabidopsis*. However, to date little is known of the TPS/TPP gene family and the function of the trehalose pathway and T6P in the regulation of carbon flow and energy status in a C_4 cereal crop such as maize.

TPS pathway is conserved in maize

The maize genome encodes families of 14 TPS and 11 TPP genes, while the TRE gene is present in a single copy, which is quite similar to what has been observed in rice, *Arabidopsis*, and poplar (reviewed by Avonce *et al.*, 2006; Lunn, 2007; Li *et al.*, 2008; Paul *et al.*, 2008; Yang *et al.*, 2012). According to evolutionary theories, the duplication process started earlier for TPS genes than for TPP genes. TPS genes have more common ancestors before the split between monocot and dicot species than do TPP genes, which were duplicated more after this event (Fig. 2). The maize class I full-length TPS gene has 16 introns, while most class II TPS genes have two introns

the night or extended dark period (recovery phase). Sucrose (A, E), glucose (B, F), fructose (C, G), and glucose derived from hydrolysed starch (D, H) was determined by capillary high pressure ion chromatography. Values are presented as means \pm SE of independent biological replicates ($n=5$).

Table 3. Correlation coefficients between sugars, starch, and T6PC

Coefficients of correlation were determined over 24 h after control (top) and shading (bottom) treatment using a Pearson comparison test ($n=3-6$). Positive and negative correlations are indicated in shades of green and red, respectively.

	Suc	Glu	Fru	Starch	T6P
Suc					
Glu					
Fru					
Starch					

	Suc	Glu	Fru	Starch	T6P
Suc					
Glu					
Fru					
Starch					

(Fig. S1), similar to rice, *Arabidopsis*, and poplar (Yang et al., 2012). Maize TPP genes have a high number of introns (7–10), with the exception of *ZmTPPB.1.2* and *-1.3*, which respectively display five and three introns (Supplementary Fig. S1 at JXB online). These results indicate a striking conservation of gene structure across species.

At least one class I TPS gene encodes a catalytic TPS. The maize genome has two class I TPS genes (Fig. 1A, Table 1), with one of them (*ZmTPSI.1.1*, also called *ZmTPSI*) encoding a functional enzyme (Jiang et al., 2010). The other (*ZmTPSI.1.2*) has a truncated TPS domain, which makes it unlikely to be functional. Since its sequence is very similar to *ZmTPSI.1.1*, it may have been duplicated recently, and its function remains unknown. Class I TPS genes also harbour a TPP domain; however, they lack some of the motifs forming the active site of phosphatase proteins belonging to the HAD superfamily (Lunn, 2007; Vandesteene et al., 2010). The maize genome encodes 12 class II TPS genes in four sub-clades (A2–A5) that include sequences from both monocots and dicots (Fig. 2A). Their structure is similar to *Arabidopsis* class II TPS genes, which have both TPS and TPP domains. As shown in Table 1, they all display both TPS and TPP domains with numerous substitutions in amino acids that are essential for substrate binding and conserved phosphatase motifs (Avonce et al., 2006; Lunn, 2007; Vandesteene et al., 2010). The role for the class II TPS enzymes has been mostly undefined (Chary et al., 2008; Singh et al., 2011); however, the class II TPS genes display remarkable differential spatial and temporal expression patterns in *Arabidopsis* (Ramon et al., 2009). To understand better the possible function for the maize class II TPS proteins, we reviewed the raw data from public genome-wide transcript analyses with attention to class II TPS genes. There were numerous instances where TPS/TPP transcripts showed remarkable spatial and temporal

Table 4. Correlation coefficients between transcripts and sugars

Coefficients of correlation were determined over 24 h after control (top) and dark (bottom) treatment using a Pearson comparison test ($n=3-6$). Positive and negative correlations are indicated in shades of green and red, respectively.

	Suc	Glu	Fru	Starch	T6P
ZmTPSI.1.1	0.02	-0.24	-0.01	-0.21	0.10
ZmTPSII.2.1	-0.88	-0.96	-0.77	-0.97	-0.65
ZmTPSII.3.2	-0.75	-0.56	-0.75	-0.55	-0.80
ZmTPSII.3.3	-0.68	-0.84	-0.72	-0.80	-0.63
ZmTPSII.4.1	-0.99	-0.93	-0.94	-0.93	-0.90
ZmTPSII.4.2	-0.70	-0.49	-0.70	-0.49	-0.76
ZmTPSII.4.3	-0.58	-0.35	-0.55	-0.36	-0.61
ZmTPSII.5.3	-0.97	-0.96	-0.99	-0.93	-0.96
ZmTPSII.5.4	-0.99	-0.95	-0.98	-0.94	-0.94
ZmTPPA.3	0.89	0.77	0.92	0.75	0.95
ZmTPPA.1	-0.75	-0.61	-0.86	-0.56	-0.93
ZmTPPB.1.3	0.52	0.29	0.57	0.28	0.67
ZmβGal	0.38	0.52	0.15	0.59	-0.02
ZmAKINβ	-0.83	-0.68	-0.87	-0.66	-0.91
ZmARG10	-0.92	-0.82	-0.95	-0.79	-0.97
ZmMDH	0.42	0.63	0.35	0.64	0.21
ZmbZIP11	0.13	-0.13	0.03	-0.08	0.10
ZmDPS	-0.59	-0.74	-0.74	-0.66	-0.71

	Suc	Glu	Fru	Starch	T6P
ZmTPSI.1.1	-0.37	-0.78	-0.67	1.00	0.82
ZmTPSII.2.1	-0.81	-0.33	-0.76	0.09	-0.46
ZmTPSII.3.2	-0.85	-0.49	-0.90	0.34	-0.22
ZmTPSII.3.3	-0.88	-0.43	-0.78	0.15	-0.43
ZmTPSII.4.1	-0.81	-0.38	-0.84	0.20	-0.35
ZmTPSII.4.2	-0.89	-0.44	-0.74	0.12	-0.47
ZmTPSII.4.3	-0.52	0.03	-0.65	-0.11	-0.50
ZmTPSII.5.3	-0.79	-0.34	-0.82	0.16	-0.38
ZmTPSII.5.4	-0.87	-0.39	-0.73	0.09	-0.49
ZmTPPA.3	-0.19	0.38	-0.39	-0.36	-0.55
ZmTPPA.1	-0.97	-0.59	-0.67	0.17	-0.45
ZmTPPB.1.3	0.99	0.70	0.78	-0.35	0.27
ZmβGal	0.35	0.34	0.90	-0.66	-0.44
ZmAKINβ	-0.73	-0.15	-0.54	-0.19	-0.70
ZmARG10	-0.79	-0.23	-0.59	-0.12	-0.65
ZmMDH	0.03	-0.59	-0.22	0.86	0.95
ZmbZIP11	0.20	-0.32	0.50	0.22	0.41
ZmDPS	0.28	-0.34	0.36	0.42	0.67

specificity; *ZmTPSII.2.2* in the ovule, *ZmTPSII.3.3*, *-4.2*, *-5.3*, and *-5.4* in the leaf, and *ZmTPSII.5.3* in the endosperm (Davidson et al., 2011; Sekhon et al., 2011). Based on the pattern of expression observed during the diurnal cycle and recovery from darkness, we suggest that the maize class II TPS enzymes play a regulatory role in responding to and/or managing energy resources in seedling leaf tissue, perhaps through its interaction with sugar phosphates.

Close examination of the substrate binding and catalytic domains of the maize class II TPS proteins suggests that this group may not possess catalytic activity (Table 1). R391 has

been shown to be required for binding UDPG in the catalytically active TPS1. Substitution of R391 with an aspartate residue in all maize class II TPS proteins may abolish enzymatic activity. This corresponds to the absence of TPS activity observed for most class II TPS in *Arabidopsis* (Vandesteene *et al.*, 2010). Maize class II genes from subclade 5 display more variations in the binding site for UDPG than genes from subclades 2, 3, and 4. Few substitutions are observed, however, in the G6P-binding site (except a minor substitution of T284S). One possible explanation is that the class II TPS proteins have lost their catalytic activity but have retained the binding site for G6P. This may indicate a sensing as opposed to a catalytic function. Such is the case for the plant pathogenic fungi *Magnaporthe grisea* TPS1 gene, which has a regulatory function in the pentose pathway required for fungal virulence. This involves G6P binding without formation of T6P, and the association with a regulator protein, TPS3 (Wilson *et al.*, 2007). Since plant and fungal trehalose pathways are somewhat similar (Avonce *et al.*, 2010), a similar process could occur in maize. The existence of high-molecular-weight TPS complexes has already been demonstrated in rice (Zang *et al.*, 2011).

Similar to TPP from other plants, maize TPPs consist of a TPP domain with three conserved phosphatase domains required for activity. Only *ZmTPPB.2.1*, also called RA3, was demonstrated as a functional TPP enzyme in maize where it controls inflorescence branching (Satoh-Nagasawa *et al.*, 2006; Carillo *et al.*, 2013). Genes belonging to subclade A2, A3, or B2 are found only in monocot species, i.e. maize, rice, or sorghum (data not shown), which means that they could have arisen later in evolution or have been lost in dicots. As with TPS genes, several TPP genes show spatial and temporal expression patterns; *ZmTPPA.3* and *ZmTPPB.1.3* in the leaf, *ZmTPPB.2.2* and *-2.3* in anthers and pollen, *ZmTPPA.3* in roots, and *ZmTPPB2.1* in the endosperm, suggesting tissue-specific functions (Davidson *et al.*, 2011; Sekhon *et al.*, 2011).

TPS/TPP genes show a diurnal pattern of expression

Diurnally expressed genes participate in growth, development, reproduction, and metabolism (Smith *et al.*, 2004; Bläsing *et al.*, 2005; Osuna *et al.*, 2007). So far, the relationship between the trehalose pathway genes and maintenance of energy balance throughout the day/night cycle is not well defined. TPS/TPP gene expression was shown in *Arabidopsis* to be sensitive to sucrose depletion (Thimm *et al.*, 2004; Lunn *et al.*, 2006). In maize, the highest mRNA levels for class II TPS genes were at the end of the regular 8 h night period, corresponding to the lowest levels of sucrose, starch, and T6P (Fig. 6A). These results agree with those seen in *Arabidopsis* (Lunn *et al.*, 2006; Wahl *et al.*, 2013). In maize seedlings subjected to a typical diurnal cycle (16 h day/8 h night), we observed that all class II TPS genes demonstrated a diurnal pattern of gene expression with transcript levels decreasing throughout the day and increasing throughout the night (Fig. 3). Interestingly, T6P levels in the same samples showed a distinct diurnal pattern with levels increasing throughout the day and decreasing throughout the night (Fig. 5). Debast *et al.* (2011) used transgenic potato tubers to produce

artificially elevated or reduced T6P levels. They observed that elevated T6P resulted in a reduction of transcripts for two class II TPS genes (*TPS8* and *TPS11*), and repressed T6P levels induced the transcription of these genes. These results in potato corroborate our observation in maize that class II TPS transcripts are inverted with respect to T6P levels throughout the diurnal cycle.

Circadian clock-regulated genes participate in a large number of physiological processes, preparing the plant for the rhythmic change in its environment. In *Arabidopsis*, as much as 30% of the expressed transcripts cycle every 24 h under constant light and temperature (Covington *et al.*, 2008). The rhythmic control of circadian-regulated genes continues after cycling environmental cues, i.e. light and temperature, have been removed (Doherty and Kay, 2010; Khan *et al.*, 2010). Kahn *et al.* (2010) set the circadian clock by exposing maize seedlings to 12 h light/12 h dark, and then switched to continuous light for 48 h to identify those genes regulated by the circadian clock. They identified >1300 transcripts that maintained a circadian rhythm even after being switched to continuous light. Here, we identified four TPS genes that were among the data they collected; *ZmTPSI.1.1* and *ZmTPSII.5.3*, *-2.1*, and *-3.2*. For these four TPS genes, transcript levels drift along with complete loss of circadian cycling. At least for these four TPS genes, it can be concluded that gene expression is not regulated by the circadian clock but rather by the energy status of the cell (Gibon *et al.*, 2004).

All of the maize TPS and TPP genes examined in this work showed some degree of diurnal cycling and, based on the results of Khan *et al.* (2010), are regulated by energy status as opposed to an internal clock. This provides further evidence that regulation of the trehalose pathway is tightly linked to sugar levels and plays an important role in maintaining sensing and energy stasis. Usadel *et al.* (2008) observed transcriptome changes in vegetative *Arabidopsis* rosettes throughout the diurnal cycle and after 4 h of extended night. Examination of their data revealed that, as with maize, under regular diurnal cycling all class II TPS genes showed diurnal cycling with highest expression at the end of the night period, with the exception of *AtTPS5*, which has no homologue in maize (Figs 2 and S3B).

In contrast to the class II genes, *ZmTPSI.1.1* is expressed at its lowest levels during the night and rises during the day, which is out of phase with sucrose and T6P levels, again in agreement with that seen with all class I genes in *Arabidopsis* (Fig. S3A) (Usadel *et al.*, 2008). Low transcript levels at the end of night and at end of the extended darkness indicate that *ZmTPSI.1.1* is not likely to be under the control of SnRK1 as with the class II genes (Fig. 6). *ZmTPSI.1.1* is perhaps regulated at the post-translational level through interaction with specific kinases, e.g. SnRK1, phosphatases, or class II TPS proteins (Gliński and Weckwerth, 2005; Harthill *et al.*, 2006). We also observed that gene expression patterns for the three predominant maize TPP genes, *ZmTPPA.3*, *ZmTPPB.1.3*, and *ZmTPPA.1*, was varied and showed unique patterns of expression. *ZmTPPA.3* and *ZmTPPB.1.3* had the lowest expression at the end of the night period, in contrast to what was observed for class II TPS transcripts, and is consistent

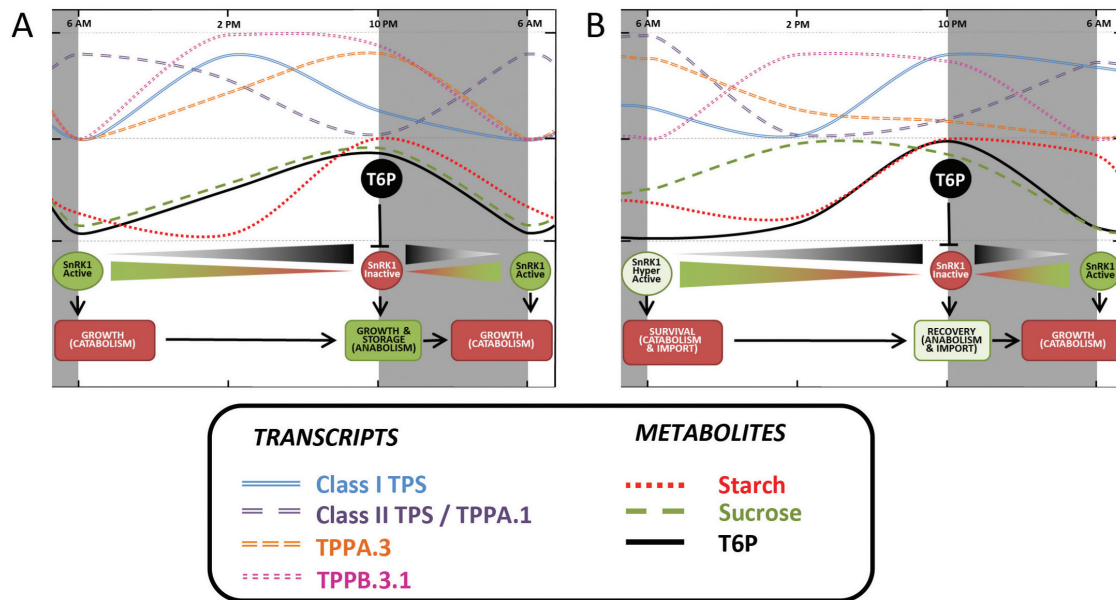


Fig. 6. Model depicting a role for the maize trehalose pathway in regulating sugar metabolism and growth under regular day/night cycles and extended darkness in juvenile maize leaves. The model is based on data from the present and previously published work (Baena-González et al., 2007; Zeeman et al., 2007; Zhang et al., 2009; Ghillebert et al., 2011; Wahl et al., 2013) (A) Under regular day/night cycles, as day proceeds, photosynthesis induces the accumulation of sucrose and T6P. Rising levels of T6P gradually promote the inactivation of SnRK1 and the redox activation of AGPase in order to turn on anabolism, growth, and starch accumulation. During the night, starch and sucrose are used as a source of carbon and energy for growth to continue. Their levels, as well as those of T6P, decrease during the night and are low in the morning. Low T6P levels induce: (i) a minimal AGPase (starch synthesis enzyme) activation, preventing starch synthesis; and (ii) a maximal activation of SnRK1 (major energy sensor), enabling growth by partially activating catabolism through starch/sugar consumption. (B) During extended darkness, sucrose and starch immediately available are rapidly used. In order to keep cell stasis, leaf cells slow down metabolism and mobilize alternative carbon sources other leaves, roots, or the attached seed. This generates an accelerated accumulation of remobilized sucrose, negatively correlating with very low T6P levels and a strong activation of SnRK1 at the end of the stress period. SnRK1 activity correlates with the induction of *ZmAKINβ* and *ZmARG10* (positive SnRK1 targets) and all the class II TPS genes, and the repression of *ZmbZIP11* and *ZmDPS* (negative SnRK1 targets) by the end of extended dark period. During recovery, 8 h after stress relief, the opposite phenomenon occurs. Photosynthesis is turned on when there remains an abundant supply of imported sucrose, while T6P levels continue to be low because of the perceived stress. Our results indicate that the cell detects intracellularly derived sucrose independently of that which is imported, possibly sensed through hexokinase. Eight hours after returning to the light, T6P levels remain low; however, they increase enough to inactivate SnRK1. Transcript levels change, with a strong repression of *ZmAKINβ* and *ZmARG10* (positive SnRK1 targets) and all the Class II TPS genes, and there is induction of *ZmbZIP11* and *ZmDPS* (negative SnRK1 targets). These SnRK1/T6P-mediated changes result in the switch from growth-from-catabolism to growth-from-anabolism. After 24–48 h of recovery, the plant goes back to its regular cycle at the transcriptional level, while metabolites levels are still being affected.

with their role in the dephosphorylation of T6P (Lunn et al., 2006). *ZmTPPA.1* followed an expression pattern similar to the class II TPS genes, with the highest expression at the end of the dark period. This variable pattern for TPP genes is the same as seen in *Arabidopsis* (Fig. S3C) (Usadel et al., 2008).

Effect of extended darkness on energy status and TPS/TPP gene expression

It has been shown previously that reducing light by as little as 30% can have significant impact on grain production and total biomass yield in maize (Earley et al., 1966). Setter et al. (2001) imposed 5 d of shade stress on flowering maize plants. They observed a 66% reduction in kernel dry matter production, along with a 20–50% reduction in floret carbohydrates. Here, we also observed that extended darkness (48 h) had a significant effect on starch and sucrose levels in maize seedlings (Fig. 4). Surprisingly, 48 h of darkness resulted in higher levels of sucrose and starch, suggesting a slowing of carbon usage for growth including cell expansion (Fig. 2) and the mobilization of carbohydrate reserves stored in other leaves, roots, or the attached seed. During a regular light/dark cycle, the plant

draws on cellular reserves of sugar and starch to fuel metabolism and growth. Our results indicate that, during prolonged darkness, the plant enters a metabolic stasis in order to survive.

Perhaps most surprising was the observation that when plants were returned to a light/dark cycle after extended darkness, T6P levels no longer followed the same time course as sucrose and hexose sugars. When *Arabidopsis* plants were subjected to extended nights (Usadel et al., 2009), during leaf senescence (Wingler et al., 2012), were starved for carbon (Yadav et al., 2014), or in the maize seedling leaf under a regular diurnal cycle (Fig. 6), T6P levels always followed the same time course as sucrose and hexose sugars. We suggest that, after a period of darkness, leaf 3 does not sense sugars imported from other parts of the plant in the same manner as cellular-derived sugar sources. Certainly the cell does not appear to sense imported sugars through T6P or SnRK1. The difference may lie in the mechanism of sucrose degradation i.e., invertase, sucrose synthase, and the products of these reactions, i.e. glucose, fructose, UDPG, or modifications in the sucrose sensing pathways.

Schussler and Westgate (1995) suggested that it is the flux of carbohydrates into the developing ovary as opposed to

sugar concentration per se that determines kernel set. A possible mechanism for sugar sensing could be through the rapidly turning over pool of intermediates such as UDPG and G6P (Setter *et al.*, 2001). These are also substrates for TPS, or are capable of binding to a catalytically inactive TPS protein. Usadel *et al.* (2009) observed the effect of extending the night by an additional 6h for maize seedlings. Using the maize 18K Affymetrix chip, they found that extending the dark (similar to short days for *Arabidopsis*) resulted in a 2- to 4-fold increase in transcripts for several class II TPS genes (*ZmTPSII.4.1*, *-5.2*, and *-5.3*), suggesting that class II TPS enzymes participate in maintaining the survival state through its sensing of sugars. We observed that all class II TPS transcripts that were typically at their highest level at 6 a.m. were several orders of magnitude higher after 48h of darkness, and dropped rapidly as sucrose levels rose in the light (Fig. 3). These results suggest an important role for the maize class II TPS enzymes in prolonging survival and in recovering from extended darkness. As before, *Arabidopsis* class II TPS genes are induced by extended night, with the exception of *AtTPS5* (Usadel *et al.*, 2008). Osuna *et al.* (2007) starved *Arabidopsis* seedlings grown in liquid culture under low light by withholding sucrose for 48h. They observed a rapid (30min) sucrose-dependent alteration in transcripts for more than 1000 genes, including a decrease in *AtTPS8*, *AtTPS9*, *AtTPS10*, and *AtTPS11*. One possible explanation for the pattern seen in Fig. 4A is that class II genes are expressed when sucrose levels are low and SnRK1 is active. This result is consistent with the transcriptional co-regulation of various TPS genes by energy related stresses (sucrose starvation, darkness, etc.), and the SnRK1 catalytic subunit KIN10 in *Arabidopsis* (Baena-González, 2007; Ghillebert *et al.*, 2011).

A very different response was observed for the catalytically active *ZmTPSI.1.1* in that extended darkness resulted in repression of the transcript at a time it normally peaks during the diurnal cycle (2 p.m.). Indeed we found that, in plants recovering from extended darkness, the *ZmTPSI.1.1* transcript closely mimicked starch levels ($R^2=99\%$). The relationship between the *ZmTPSI.1.1* transcript and starch levels after extended darkness indicates a metabolic shift from short-term sugar depletion (8h) to long-term absence of photosynthate (48h) with a possible role for starch in the formation, hydrolysis, and/or sensing to regulate *ZmTPSI.1.1* levels. This hypothesis could be supported by the results of Scialdone *et al.* (2013), indicating that *Arabidopsis* plants sense both starch and day length in order to regulate starch degradation rate. Such a phenomenon could be involved in regulation of target metabolic genes to enable the plant to adjust its environment according to its internal resources.

Role of TPS, TPP, and T6P in sugar sensing and maintenance of energy status

Based on literature from *Arabidopsis* and potato, as well as our results, we present a model for the regulation of energy balance throughout the diurnal cycle and the recovery from extended darkness that incorporates the trehalose pathway genes T6P and SnRK1 (Fig. 6). During a typical night

period, starch is consumed to maintain growth and cellular metabolism. Starch breakdown provides less sucrose than carbon fixation in the light, and thus sucrose levels fall as reflected by a decrease in T6P levels, with a peak at dusk and a minimum at dawn (Lunn *et al.*, 2006; Wahl *et al.*, 2013). This occurs coincidentally with an observed increase in transcription of SnRK1 target (inducible) genes (Usadel *et al.*, 2008) (Supplementary Fig. S3D, E). AGPase is then inactivated by changes in allosteric regulators and by light- and sucrose-dependent post-translational redox modification, while starch degradation is stimulated (Tiessen *et al.*, 2003; Gibon *et al.*, 2004; Kolbe *et al.*, 2005; Lunn *et al.*, 2006). SnRK1 activity is also induced during the night as the plant enters sink mode (Baena-González *et al.*, 2007), correlated with the transcription of class II TPS genes in maize, such that their peak expression is at the end of the night period, and this may result in SnRK1-mediated phosphorylation of some TPS1 (Glinski and Weckwerth, 2005), resulting in feedback regulation of the trehalose pathway. Upon re-illumination, sucrose and starch accumulate and T6P levels rise (Wahl *et al.*, 2013), inhibiting SnRK1 (Zhang *et al.*, 2009). This is accompanied by the activation of AGPase, repression of starch degradation, upregulation of *ZmTPSI.1.1* gene expression, and downregulation of class II TPS transcription. These transcriptional and metabolic changes are consistent with cell growth, with its optimum at the end of the day.

Interestingly, in maize seedling leaf tissue, each of the eight class II TPS genes showed the same pattern of transcript induction during the night, although these genes show quite varied expression throughout development and in response to environmental stimuli (Covington *et al.*, 2008; Wahl *et al.*, 2013). The only maize TPS gene known to have catalytic function, *ZmTPSI.1.1*, was not induced after extended darkness; however, was induced during the afternoon. Extended darkness resulted in even lower transcript levels for *ZmTPSI.1.1*, an indication that transcriptional regulation of this TPS gene is critical during normal growth and not while the plant is subjected to prolonged darkness. One can infer from this that: (i) *ZmTPSI.1.1* expression does not require SnRK1 to be active; or (ii) its transcriptional regulation is not important in the production of stress-induced T6P. Our attention now turns to the class II TPS genes in maize in regard to their role in sugar metabolism and during the recovery from extended darkness.

Conclusions

The maize family of trehalose biosynthetic enzymes offers a fascinating system for the characterization of energy management with respect to sucrose and starch, and how it contributes to crop productivity and stress tolerance. Regarding the present results, recovery from extended darkness probably involved the participation of class II TPS proteins. It is of great interest to determine their role in this process, whether they can function catalytically alone or as regulatory elements of a high-molecular-weight complex, or if they act as signaling molecules or transcription factors. Further investigation into protein-protein interactions will validate this hypothesis.

The use of mutants and transgenic plants will facilitate our understanding of how each TPS and TPP enzyme contributes to what is undoubtedly a complex regulatory apparatus. We also observed that extended darkness disrupted the connection between sucrose and T6P, suggesting multiple sucrose sensing pathways operating simultaneously.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Methods S1. Methods for sugars and starch analysis.

Supplementary Method S2. Formulae for sugar and starch (glucose) analysis.

Supplementary Table S1. Gene names and accession numbers for maize, rice, *Arabidopsis*, and poplar TPS and TPP.

Supplementary Table S2. Sequence of primers used for quantitative PCR.

Supplementary Fig. S1. Gene structures with introns for maize TPS I and II genes.

Supplementary Fig. S2. Predicted enzymatic domains for maize TPS and TPP genes.

Supplementary Fig. S3. *Arabidopsis* gene expression

Acknowledgements

Authors would like to thank Drs Harkamal Walia and James Specht/George Graef laboratories for sharing their equipment to grind tissue samples and perform quantitative PCR analysis with us. We also want to thank Drs Soulaïman Sakr (Agrocampus-ouest, INHP) and John Lunn (Max Planck Institute) for their time and valuable comments to significantly improve the manuscript.

References

- Ali Q, Ashraf M.** 2011. Induction of drought tolerance in maize (*Zea mays* L.) due to exogenous application of trehalose: growth, photosynthesis, water relations and oxidative defence mechanism. *Journal of Agronomy and Crop Science* **197**, 258–271.
- Avonce N, Mendoza-Vargas A, Morett E, Iturriaga G.** 2006. Insights on the evolution of trehalose biosynthesis. *BMC Evolutionary Biology* **6**, 109–124.
- Avonce N, Wuyts J, Verschooten K, Vandesteene L, Van Dijck P.** 2010. The *Cytophaga hutchinsonii* ChTPSP: first characterized bifunctional TPS-TPP protein as putative ancestor of all eukaryotic trehalose biosynthesis proteins. *Molecular Biology and Evolution* **27**, 359–369.
- Baena-González E, Rolland F, Thevelein JM, Sheen J.** 2007. A central integrator of transcription networks in plant stress and energy signalling. *Nature* **448**, 938–942.
- Bläsing O, Gibon Y, Günther M, Hohne M, Morcuende R, Osuna D, Thimm O, Usadel B, Scheible W-R, Stitt M.** 2005. Sugars and circadian regulation make major contributions to the global regulation of diurnal gene expression in *Arabidopsis*. *Plant Cell* **17**, 3257–3281.
- Brouquisse R, Gaudillere J, Raymond P.** 1998. Induction of a carbon-starvation-related proteolysis in whole maize plants submitted to light/dark cycles and to extended darkness. *Plant Physiology* **117**, 1281–91.
- Carillo P, Feil R, Gibon Y, Satoh-Nagasawa N, Jackson D, Bläsing OE, Stitt M, Lunn JE.** 2013. A fluorometric assay for trehalose in the picomole range. *Plant Methods* **9**, 21.
- Chary SN, Hicks GR, Choi YG, Carter D, Raikhel N. V.** 2008. Trehalose-6-phosphate synthase/phosphatase regulates cell shape and plant architecture in *Arabidopsis*. *Plant Physiology* **146**, 97–107.
- Covington MF, Maloof JN, Straume M, Kay SA, Harmer SL.** 2008. Global transcriptome analysis reveals circadian regulation of key pathways in plant growth and development. *Genome Biology* **9**, R130.
- Davidson RM, Hansey CN, Gowda M, et al.** 2011. Utility of RNA sequencing for analysis of maize reproductive transcriptomes. *Plant Genome Journal* **4**, 191.
- Debast S, Nunes-nesi A, Hajirezaei MR, Hofmann J, Sonnewald U, Fernie AR, Bornke F.** 2011. Altering trehalose-6-phosphate content in transgenic potato tubers affects tuber growth and alters responsiveness to hormones during sprouting 1. *Plant Physiology* **156**, 1754–1771.
- Delatte TL, Sedijani P, Kondou Y, Matsui M, de Jong GJ, Somsen GW, Wiese-Klinkenberg A, Primavesi LF, Paul MJ, Schluempmann H.** 2011. growth arrest by trehalose-6-phosphate: an astonishing case of primary metabolite control over growth by way of the SnRK1 signaling pathway. *Plant Physiology* **157**, 160–174.
- Doherty CJ, Kay SA** 2010. Circadian control of global gene expression patterns. *Annual Review of Genetics* **44**, 419–44.
- Earley EB, Miller RJ, Reichert GL, Hageman RH.** 1966. Effects of shade on maize production under field conditions. **6**, 1–7.
- Ghillebert R, Swinnen E, Wen J, Vandesteene L, Ramon M, Norga K, Rolland F, Winderickx J.** 2011. The AMPK/SNF1/SnRK1 fuel gauge and energy regulator: structure, function and regulation. *FEBS Journal* **278**, 3978–3990.
- Gibon Y, Bläsing OE, Palacios-Rojas N, Pankovic D, Hendriks JHM, Fisahn J, Höhne M, Günther M, Stitt M.** 2004. Adjustment of diurnal starch turnover to short days: depletion of sugar during the night leads to a temporary inhibition of carbohydrate utilization, accumulation of sugars and post-translational activation of ADP-glucose pyrophosphorylase in the followin. *The Plant Journal* **39**, 847–862.
- Gibon Y, Pyl E-T, Sulpice R, Lunn JE, Höhne M, Günther M, Stitt M.** 2009. Adjustment of growth, starch turnover, protein content and central metabolism to a decrease of the carbon supply when *Arabidopsis* is grown in very short photoperiods. *Plant, Cell & Environment* **32**, 859–74.
- Gibson RP, Turkenburg JP, Charnock SJ, Lloyd R, Davies GJ.** 2002. Insights into trehalose synthesis provided by the structure of the retaining glucosyltransferase OtsA. *Chemistry & Biology* **9**, 1337–46.
- Glinski M, Weckwerth W.** 2005. Differential multisite phosphorylation of the trehalose-6-phosphate synthase gene family in *Arabidopsis thaliana*: a mass spectrometry-based process for multiparallel peptide library phosphorylation analysis. *Molecular & Cellular Proteomics* **4**, 1614–1625.
- Harmer SL, Hogenesch JB, Straume M, Chang H-S, Han B, Zhu T, Wang X, Kreps JA, Kay SA.** 2000. Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. *Science* **290**, 2110–2113.
- Harthill JE, Meek SEM, Morrice N, Peggie MW, Borch J, Wong BHC, Mackintosh C.** 2006. Phosphorylation and 14-3-3 binding of *Arabidopsis* trehalose-phosphate synthase 5 in response to 2-deoxyglucose. *The Plant Journal* **47**, 211–223.
- Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J.** 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biology* **8**, R19.
- Jiang W, Fu F-L, Zhang S-Z, Wu L, Li W-C.** 2010. Cloning and characterization of functional trehalose-6-phosphate synthase gene in maize. *Journal of Plant Biology* **53**, 134–141.
- Khan S, Rowe SC, Harmon FG.** 2010. Coordination of the maize transcriptome by a conserved circadian clock. *BMC Plant Biology* **10**, 126–141.
- Kolbe A, Tiessen A, Schluempmann H, Paul M, Ulrich S, Geigenberger P.** 2005. Trehalose 6-phosphate regulates starch synthesis via posttranslational redox activation of ADP-glucose pyrophosphorylase. *Proceedings of the National Academy of Sciences, USA* **102**, 11118–11123.
- Li P, Ma S, Bohnert HJ.** 2008. Coexpression characteristics of trehalose-6-phosphate phosphatase subfamily genes reveal different functions in a network context. *Physiologia Plantarum* **133**, 544–56.
- Lunn JE.** 2007. Gene families and evolution of trehalose metabolism in plants. *Functional Plant Biology* **34**, 550.
- Lunn JE, Delorge I, Figueroa CM, Van Dijck P, Stitt M.** 2014. Trehalose metabolism in plants. *The Plant Journal* **49**, n/a–n/a.

- Lunn JE, Feil R, Hendriks JHM, Gibon Y, Morcuende R, Osuna D, Scheible W-R, Carillo P, Hajirezaei M-R, Stitt M. 2006. Sugar-induced increases in trehalose 6-phosphate are correlated with redox activation of ADP-glucose pyrophosphorylase and higher rates of starch synthesis in *Arabidopsis thaliana*. *Biochemical Journal* **397**, 139–148.
- Ma J, Hanssen M, Lundgren K, et al. 2011. The sucrose-regulated *Arabidopsis* transcription factor bZIP11 reprograms metabolism and regulates trehalose metabolism. *New Phytologist* **191**, 733–45.
- Martínez-Barajas E, Delatte T, Schluepmann H, de Jong GJ, Somsen GW, Nunes C, Primavesi LF, Coello P, Mitchell RAC, Paul MJ. 2011. Wheat grain development is characterized by remarkable trehalose 6-phosphate accumulation pregrain filling: tissue distribution and relationship to SNF1-related protein kinase1 activity. *Plant Physiology* **156**, 373–381.
- Mutisya J, Sun C, Jansson C. 2009. Circadian oscillation of starch branching enzyme gene expression in the sorghum endosperm. *Plant Signaling & Behavior* **4**, 871–872.
- Nunes C, O'Hara LE, Primavesi LF, Delatte TL, Schluepmann H, Somsen GW, Silva AB, Fevereiro PS, Wingler A, Paul MJ. 2013. The trehalose 6-phosphate/SnRK1 signaling pathway primes growth recovery following relief of sink limitation. *Plant Physiology* **162**, 1720–32.
- Osuna D, Usadel B, Morcuende R, et al. 2007. Temporal responses of transcripts, enzyme activities and metabolites after adding sucrose to carbon-deprived *Arabidopsis* seedlings. *The Plant Journal* **49**, 463–91.
- Paul M. 2007. Trehalose 6-phosphate. *Current Opinion in Plant Biology* **10**, 303–9.
- Paul MJ, Primavesi LF, Jhurreea D, Zhang Y. 2008. Trehalose metabolism and signaling. *Annual Review of Plant Biology* **59**, 417–441.
- Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29**, e45.
- Polge C, Thomas M. 2007. SNF1/AMPK/SnRK1 kinases, global regulators at the heart of energy control? *Trends in Plant Science* **12**, 20–28.
- Ponnu J, Wahl V, Schmid M. 2011. Trehalose-6-phosphate: connecting plant metabolism and development. *Frontiers in Plant Science* **2**, 70.
- Ramon M, De Smet I, Vandesteene L, Naudts M, Leyman B, Van Dijck P, Rolland F, Beeckman T, Thevelein JM. 2009. Extensive expression regulation and lack of heterologous enzymatic activity of the Class II trehalose metabolism proteins from *Arabidopsis thaliana*. *Plant, Cell & Environment* **32**, 1015–1032.
- Rodríguez-Salazar J, Suárez R, Caballero-Mellado J, Iturriaga G. 2009. Trehalose accumulation in *Azospirillum brasilense* improves drought tolerance and biomass in maize plants. *FEMS Microbiology Letters* **296**, 52–59.
- Satoh-Nagasawa N, Nagasawa N, Malcomber S, Sakai H, Jackson D. 2006. A trehalose metabolic enzyme controls inflorescence architecture in maize. *Nature* **441**, 227–230.
- Schluepmann H, Berke L, Sanchez-Perez GF. 2011. Metabolism control over growth: a case for trehalose-6-phosphate in plants. *Journal of Experimental Botany* **63**, 3379–3390.
- Schussler JR, Westgate ME. 1995. Assimilate flux determines kernel set at low water potential in maize. *Crop Science* **35**, 1074–1080.
- Sciences B, Zeid IM. 2009. Trehalose as osmoprotectant for maize under salinity-induced stress. *Research Journal of Agriculture and Biological Sciences* **5**, 613–622.
- Sekhon RS, Lin H, Childs KL, Hansey CN, Buell CR, de Leon N, Kaepler SM. 2011. Genome-wide atlas of transcription during maize development. *The Plant Journal* **66**, 553–563.
- Setter TL, Flannigan BA, Melkonian J. 2001. Loss of kernel set due to water deficit and shade in maize. *Crop Science* **41**, 1530.
- Sheen J. 2010. Discover and connect cellular signaling. *Plant Physiology* **154**, 562–566.
- Singh V, Louis J, Ayre BG, Reese JC, Shah J. 2011. TREHALOSE PHOSPHATE SYNTHASE11-dependent trehalose metabolism promotes *Arabidopsis thaliana* defense against the phloem-feeding insect *Myzus persicae*. *The Plant Journal* **67**, 94–104.
- Smith S, Fulton D, Chia T, Thorneycroft D, Chapple A, Dunstan H, Hylton C, Zeeman S, Smith A. 2004. Diurnal Changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in *Arabidopsis*. *Plant Physiology* **136**, 2687–2699.
- Stitt M, Zeeman SC. 2012. Starch turnover: pathways, regulation and role in growth. *Current Opinion in Plant Biology* **15**, 282–92.
- Sulpice R, Flis A, Ivakov AA, Apelt F, Krohn N, Encke B, Abel C, Feil R, Lunn JE, Stitt M. 2014. *Arabidopsis* coordinates the diurnal regulation of carbon allocation and growth across a wide range of photoperiods. *Molecular Plant* **7**, 137–155.
- Sulpice R, Trenkamp S, Steinfaß M, et al. 2010. Network analysis of enzyme activities and metabolite levels and their relationship to biomass in a large panel of *Arabidopsis* accessions. *Plant Cell* **22**, 2872–2893.
- Thimm O, Bläsing O, Gibon Y, Nagel A, Meyer S, Krüger P, Selbig J, Müller LA, Rhee SY, Stitt M. 2004. Mapman: a user-driven tool to display genomics data sets onto diagrams of metabolic pathways and other biological processes. *The Plant Journal* **37**, 914–939.
- Tiessen A, Prescha K, Branscheid A, Palacios N, McKibbin R, Halford NG, Geigenberger P. 2003. Evidence that SNF1-related kinase and hexokinase are involved in separate sugar-signalling pathways modulating post-translational redox activation of ADP-glucose pyrophosphorylase in potato tubers. *The Plant Journal* **35**, 490–500.
- Usadel B, Bläsing OE, Gibon Y, Retzlaff K, Höhne M, Günther M, Stitt M. 2008. Global transcript levels respond to small changes of the carbon status during progressive exhaustion of carbohydrates in *Arabidopsis* rosettes. *Plant Physiology* **146**, 1834–61.
- Usadel B, Poree F, Nagel A, Lohse M, Czedik-Eysenberg A, Stitt M. 2009. A guide to using MapMan to visualize and compare Omics data in plants: a case study in the crop species, maize. *Plant, Cell & Environment* **32**, 1211–29.
- Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paeppe A, Speleman F. 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology* **3**, research0034.1–research0034.11.
- Vandesteene L, López-Galvis L, Vanneste K, et al. 2012. Expansive evolution of the trehalose-6-phosphate phosphatase gene family in *Arabidopsis*. *Plant Physiology* **160**, 884–896.
- Vandesteene L, Ramon M, Le Roy K, Van Dijck P, Rolland F. 2010. A single active trehalose-6-P synthase (TPS) and a family of putative regulatory TPS-like proteins in *Arabidopsis*. *Molecular Plant* **3**, 406–19.
- Wahl V, Ponnu J, Schlereth A, Arrivault S, Langenecker T, Franke A, Feil R, Lunn JE, Stitt M, Schmid M. 2013. Regulation of flowering by trehalose-6-phosphate signaling in *Arabidopsis thaliana*. *Science* **339**, 704–7.
- Wilson RA, Jenkinson JM, Gibson RP, Littlechild JA, Wang Z-Y, Talbot NJ. 2007. Tps1 regulates the pentose phosphate pathway, nitrogen metabolism and fungal virulence. *EMBO Journal* **26**, 3673–85.
- Wingler A. 2002. The function of trehalose biosynthesis in plants. *Planta* **60**, 437–440.
- Wingler A, Delatte TL, O'Hara LE, Primavesi LF, Jhurreea D, Paul MJ, Schluepmann H. 2012. Trehalose 6-phosphate is required for the onset of leaf senescence associated with high carbon availability. *Plant Physiology*. doi: 10.1104/pp.111.191908.
- Yadav UP, Ivakov A, Feil R, et al. 2014. The sucrose-trehalose 6-phosphate (Tre6P) nexus: specificity and mechanisms of sucrose signalling by Tre6P. *Journal of Experimental Botany* **65**, 1051–68.
- Yang H-L, Liu Y-J, Wang C-L, Zeng Q-Y. 2012. Molecular evolution of trehalose-6-phosphate synthase (TPS) gene family in *Populus*, *Arabidopsis* and rice. *PLoS One* **7**, e42438.
- Zang B, Li H, Li W, Deng XW, Wang X. 2011. Analysis of trehalose-6-phosphate synthase (TPS) gene family suggests the formation of TPS complexes in rice. *Plant Molecular Biology* **76**, 507–522.
- Zeeman SC, Smith SM, Smith AM. 2007. The diurnal metabolism of leaf starch. *Biochemical Journal* **401**, 13–28.
- Zhang Y, Primavesi LF, Jhurreea D, Andralojc PJ, Mitchell RAC, Powers SJ, Schluepmann H, Delatte T, Wingler A, Paul MJ. 2009. Inhibition of SNF1-related protein kinase1 activity and regulation of metabolic pathways by trehalose-6-phosphate. *Plant Physiology* **149**, 1860–1871.

Supplemental Figures S1, S2, and S3

TITLE: The Trehalose Pathway in Maize: Conservation and Gene Regulation in Response to Diurnal Cycle and Extended Darkness

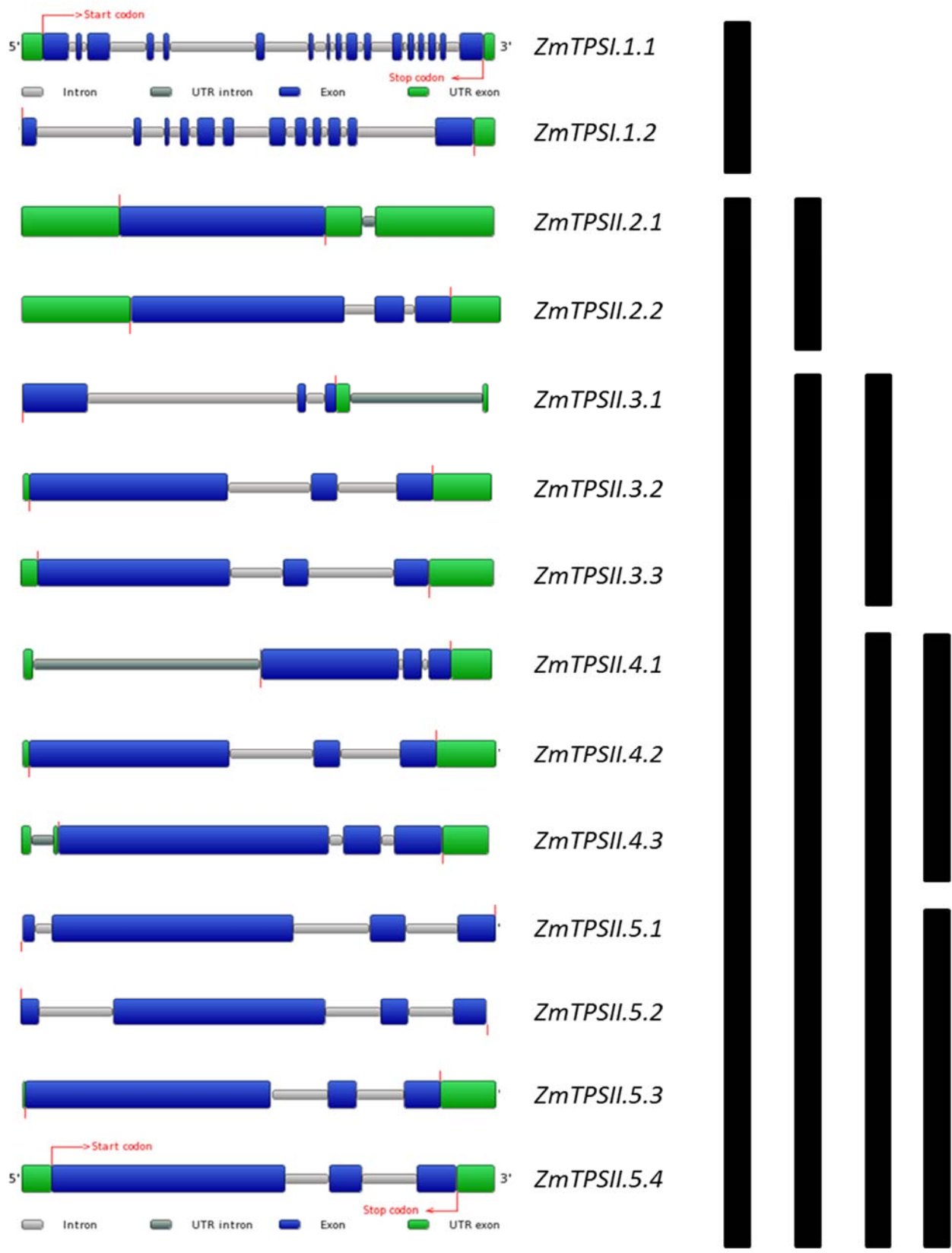
AUTHORS: Clémence Henry, Samuel W. Bledsoe, Allison Siekman, Alec Kollman, Brian M. Waters, Regina Feil, Mark Stitt and L. Mark Lagrimini

Supplemental Figure Legends

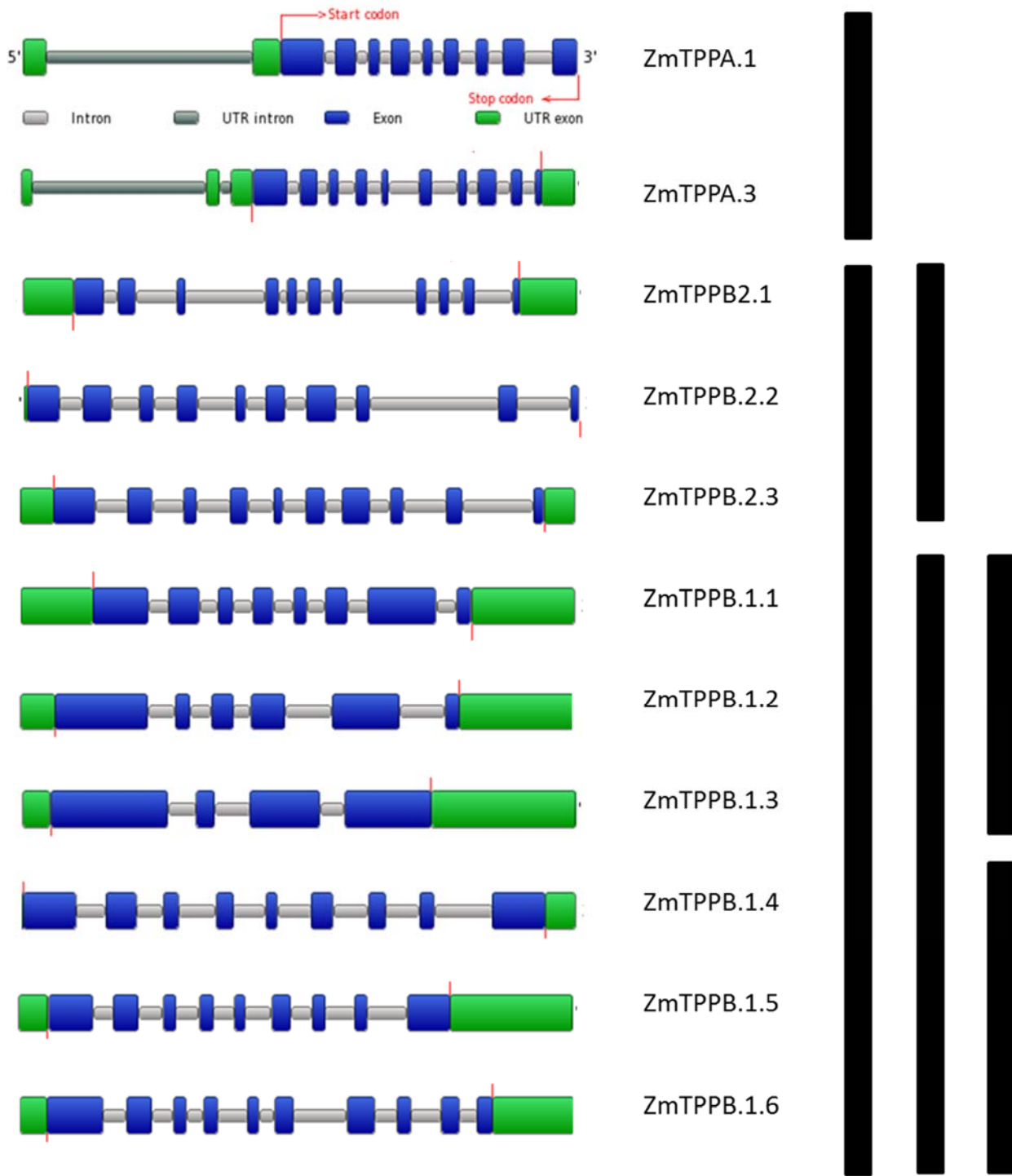
Figure S1. Gene structures with introns for maize TPS I and II genes (A) and TPP genes (B). Graphical representation of maize TPS genes showing exons (blue), introns (gray), and non-coding sequences (green).

Figure S2. Predicted enzymatic domains for maize TPS (A) and TPP (B) genes. Conserved residues for substrate binding are highlighted in red.

Figure S3. Expression of Arabidopsis (A) class I TPS genes, (B) class II TPS genes, (C) TPP genes, (D) SNRK1 (inducible) targets, (E) SnRK1 (repressible) targets in mature leaf tissue throughout the diurnal cycle and after 4 h extended night.



Supplemental Figure S1A: Gene structures with introns for maize TPS class I and class II genes.



Supplemental Figure S1B: Gene structures with introns for maize TPP class A and class B genes.

Supplemental Figure S2A: Predicted enzymatic domains for TPS proteins.

TPS predicted domain; Conserved residues **UDPG (donor)** & **G6P (acceptor)** binding

TPP predicted domain; Conserved motifs **HAD phosphatases**

ZmTPSI.1.1_tps1/1-960 MSSDAARGQ-----RGINCTRGDAAAMP TSSPFVGDGSGGAGSPIRVERMVRERSRR
ZmTPSI.1.2/1-576 -----
ZmTPSII.2.1/1-888 ---MVSRSY-----SNLLELA-----AGGSGGEP-LPSLG--RRRIPRV
ZmTPSII.2.2/1-863 ---MASRSY-----SNLLDLA-----TGAADQAPAVAALGALRRRLPRV
ZmTPSII.3.1/1-912 ---MMSRSY-----TNLLDLAEGNFAALGP---VGGSGRQR-HGSFG--LRRMSRV
ZmTPSII.3.2/1-912 ---MMSRSY-----TNLLDLAEGNFAALGP---AGGSGRQR-HGSFG--LRRMSRV
ZmTPSII.3.3/1-912 ---MMSRSY-----TNLLDLAEGNFAALGPAAGAGGGGRQR-QGSFG--LRRMSRV
ZmTPSII.4.1/1-864 ---MVLKSH-----TNLLDMCCE-----DVFDQQP-----LRSRPHV
ZmTPSII.4.2/1-865 ---MVSKEY-----SNLLDLTSG-----DGFDFRQP-----FKSLPRV
ZmTPSII.4.3/1-868 ---MVSKEY-----SNLLDMTPG-----DGFDFRRP-----FKSLPRV
ZmTPSII.5.1/1-953 MEAGASSRYPLWTRGGVQTRMEGMGGVWKRARM--EGRRRRMAVGFAGGDLGRGARE
ZmTPSII.5.2/1-860 -----MTCLGHSCGPR-----ASGPCRAAIWPFVG--VQQINLK
ZmTPSII.5.3/1-986 ---MPSFPR-----ANVVDKTRGPSPCAHAVTPPTLTSGSPRCPFLPRSDTHT
ZmTPSII.5.4/1-851 ---MPSISC-----HNLLDLA-----AADDVPLP-----SPTPLRRLPRV

ZmTPSI.1.1_tps1/1-960 YDIFASDAMDTDAEAAFALDGVQSPGRA-----SPAN
ZmTPSI.1.2/1-576 -----
ZmTPSII.2.1/1-888 -----VTASGIV-----PDLD
ZmTPSII.2.2/1-863 -----VTPGLI-----DD--
ZmTPSII.3.1/1-912 -----MTVPGTL-----TELD
ZmTPSII.3.2/1-912 -----MTVPGTL-----SELD
ZmTPSII.3.3/1-912 -----MTVPGTL-----SELD
ZmTPSII.4.1/1-864 -----VNSPGII-----SDPD
ZmTPSII.4.2/1-865 -----VTSPGII-----SDTD
ZmTPSII.4.3/1-868 -----VTSPSII-----SDHD
ZmTPSII.5.1/1-953 GGHRALCAYLENKKSGPGWKIPLPACCAPALPSRGGPRPAVKYYDPIPRFQPHENPKSQ
ZmTPSII.5.2/1-860 -----ATTGEVM-----
ZmTPSII.5.3/1-986 HIQLAIFVPPVLLPCPRPTSAFLSPETS-----AKVIDPSPTPTPTDRPPNE
ZmTPSII.5.4/1-851 -----MSVA-----

ZmTPSI.1.1_tps1/1-960 MEDAGGAAA-----
ZmTPSI.1.2/1-576 -----
ZmTPSII.2.1/1-888 VSD-----
ZmTPSII.2.2/1-863 -----
ZmTPSII.3.1/1-912 GEDESEPAA-----
ZmTPSII.3.2/1-912 GEDESEPAA-----
ZmTPSII.3.3/1-912 GEDESEPAA-----
ZmTPSII.4.1/1-864 WES-----
ZmTPSII.4.2/1-865 WDT-----
ZmTPSII.4.3/1-868 WDS-----
ZmTPSII.5.1/1-953 TKRPNPCAA-----
ZmTPSII.5.2/1-860 -----

ZmTPSII.5.3/1-986 P DTPRRVAALLFSSCAIAAVSTSATPTDRQEEDDEAMP SLSCHNLLDLAAAEVPLPSPT
ZmTPSII.5.4/1-851 -----

ZmTPSI.1.1_tps1/1-960 -----ARPLAGSRSGFRRLGLRGMKQRLLVVANRLPVSANRRGED-----HWSL
ZmTPSI.1.2/1-576 -----
ZmTPSII.2.1/1-888 -----AASAADQSS-----HAPRERVIIVANQLPVRASRRAAGAGGGWDF
ZmTPSII.2.2/1-863 -----SPASPS-----TPPRPTIIVANQLPIRSHRPESPEE---P WTF
ZmTPSII.3.1/1-912 -----TSSVADVPS-----SVAADRILIVSNQLPIVARRRPDGR----GWSF
ZmTPSII.3.2/1-912 -----TSSVADVPS-----SVAADRILIVSNQLPIVARRRPDGR----GWSF
ZmTPSII.3.3/1-912 -----TSSVADAPS-----SVAADRILIVSNQLPIVARRRPDGR----GWSF
ZmTPSII.4.1/1-864 -----SNDGNSVGS-----MPFCFKRKIIIVANFLPVICAKNEATG----EWSF
ZmTPSII.4.2/1-865 -----ISDGDSVGS-----ASSTERKIIIVANFLPLNCTRDET G----VLSF
ZmTPSII.4.3/1-868 -----ISDGDSVGS-----AFSIERKIIIVANFLPLNCTRDET G----ELSF
ZmTPSII.5.1/1-953 -----QAAAMLSV SASDGRSPCV EARRIVVTHRLPLHAEPNPDAPY---GFDF
ZmTPSII.5.2/1-860 -----VSKTSSPSASNGELSTSPVEARRIVVTYRLPLRAEPNLDSPH---GFDF
ZmTPSII.5.3/1-986 PLRLPRVMSVASPASPTSPS-----PPAPRRRVI VSHRLPLRASPDPSAPF---GFRF
ZmTPSII.5.4/1-851 -----SPASPTSP-----APAPRRRVI VSHRLPLRASPDPAAPF---GFAF

ZmTPSI.1.1_tps1/1-960 EISAGGLV SALL-----GVKDVDAKWIGWAGVNV PDEVGQRALTKALAEK-RCIPVFL
ZmTPSI.1.2/1-576 -----
ZmTPSII.2.1/1-888 AWDQDSLLLQVKDSLRAHHGRADVEFVYVGGLRDDVPP-AEHDQVAHDLLEGFRCVPTFL
ZmTPSII.2.2/1-863 EWEDDSLRLHLH-----HSSSPLMEFIYIGCLRDDIPQ-AEQDAVAQALLETHNCVPAFL
ZmTPSII.3.1/1-912 SWDDDSL LQLR-----DGIPDEMEVLFVGS LRADVPA-AEQDAVSQALLDRFRCAPVFL
ZmTPSII.3.2/1-912 SWDGD SLLQLR-----DGIPDEMEVLFVGS LRADVPA-AEQDEVSQTL LDRFRCAPVFL
ZmTPSII.3.3/1-912 VWDDDSL LQLR-----DGIPDEMEVLFVGS LRADVPV-AEQDEVSQALLDRFRCAPVFL
ZmTPSII.4.1/1-864 AMDDNQLLVQLK-----DGFPIGNEVIYVGS LNVQVDP-IEQDRVSQKLFKEHRCVPTFL
ZmTPSII.4.2/1-865 SLDHDALLMQLK-----DSFSNETDVVYVGS LKVQVDP-GEQDQVAQKLLREYRCIPTFL
ZmTPSII.4.3/1-868 SLDHDSLLMQLK-----DGFSNETDAVYVGS LKVHVDP-REQDQVAQKLLREYRCIPTFL
ZmTPSII.5.1/1-953 SLDADALPLQLA-----RGLPR--PVVFGALPSAAASISASEE LADLLARFGCSPVFL
ZmTPSII.5.2/1-860 SLDADALPLQCT-----RGLPR--PVVFGALPSAAASISESDDLADL FTRFACSPVFL
ZmTPSII.5.3/1-986 SVDAGTVAYQLR-----SGLPTNAPVLHIGTLPASAAE-AASDELSNYLLANFSCLPVYL
ZmTPSII.5.4/1-851 SVDAGTVAYQLR-----SGLPANAPVLHIGTLPAAAAE-AASDELSDYLLANFSCLPVYL

ZmTPSI.1.1_tps1/1-960 DEEIVHQY YNGYCNNILWPL--FHYLGLPQEDRL-----ATTRNFESQF
ZmTPSI.1.2/1-576 -----
ZmTPSII.2.1/1-888 PADLRSRFYHGFCCKQLWPL--FHYM-LPLSPEL-----GGRFDRLW
ZmTPSII.2.2/1-863 PTDIAERY YHGFCCKQHLWPL--FHYM-LPLSPDL-----GGRFDRLW
ZmTPSII.3.1/1-912 PDHLNDRFYHGFCCKRQLWPL--FHYM-LPFSSPASASAAATSSSVATSSPGNGCFDRSAW
ZmTPSII.3.2/1-912 PDHLNDRFYHGFCCKRQLWPL--FHYM-LPFSSPASASAAATSSSVATSSPGNGFRDRSAW
ZmTPSII.3.3/1-912 PDRLNDRFYHGFCCKRQLWPL--FHYM-LPFSSA---SAAGTTSSSAATCNGRFRDRSAW
ZmTPSII.4.1/1-864 PAELQHQFYHIFCKQHLWPL--FHYM-LPVCHDK-----DELFD RSLF
ZmTPSII.4.2/1-865 PSDLQQQFYHGFCCKQLWPL--FHYM-LPICLDK-----GELFD RSLF
ZmTPSII.4.3/1-868 PSDLQQQFYHGFCCKQLWPL--FHYM-LPICLDK-----GELFD RTLF
ZmTPSII.5.1/1-953 DPGLHKDFYDGFCKRYLWPM--LHYL-LPFTLTP-----FFGSGGLKFKANLY
ZmTPSII.5.2/1-860 DPSLHNDFYNSFCCKRYLWPILQLQYL-LPFTRSS-----DSGCLSFNEDLY
ZmTPSII.5.3/1-986 PTDLHHRFYHGFCCKHYLWPL--LHYL-LPLTPSS-----LGGLPFQRTLY

ZmTPSII.5.4/1-851 PTDLHHRFYHGFKHYLWPL--LHYL-LPLTPSS-----LGGLPFQRTLY

ZmTPSI.1.1_tps1/1-960 DAYKRANQMFAADVVEHYQ-DGDVIWCHDYHLMFLPKCLKDHDINMKVGWFLHTPFPSSE

ZmTPSI.1.2/1-576 -----

ZmTPSII.2.1/1-888 QAYVSVNKIFADKILEVISPEDEFVWVHDYHLMVLPFTFLRKRFRNRVKLGFFLHSPFPSSSE

ZmTPSII.2.2/1-863 QAYVSANKIFADKVLEVINPDDDFVWVHDYHLMVLPFTFLRKRFRNRKLGFFLHSPFPSSSE

ZmTPSII.3.1/1-912 EAYVLANKFFFEKVVEVINPEDDYVWVHDYHLLALPTFLRRRFRNLRIGFFLHSPFPSSSE

ZmTPSII.3.2/1-912 EAYVLANKFFFEKVVEVINPEDDYVWVHDYHLLALPTFLRRRFRNLRIGFFLHSPFPSSSE

ZmTPSII.3.3/1-912 EAYVLANKFFFEKVVEVINPEDDYVWVHDYHLMALPTFLRRCFNLRIGFFLHSPFPSSSE

ZmTPSII.4.1/1-864 QAYVRANKIFADKIVEAVNSDDDCVWVHDYHMLLIPTLLRKKLHRIKVGFFLHSPFPSSSE

ZmTPSII.4.2/1-865 QAYVRANKLIFADKVMEAINADDFVWVHDYHMLLPTFLRKRRLHRIKIGFFLHSPFPSSSE

ZmTPSII.4.3/1-868 QAYVRANKLIFADKVMEAINDDDYVWVHDYHMLLPTFLRKRRLHRIKIGFFLHSPFPSSSE

ZmTPSII.5.1/1-953 RAYLTANTQYAEVLEQLNPEDELVFIHDYHLLALPTILRHKSPRARIGFFLHTPFPTSE

ZmTPSII.5.2/1-860 RAYLTANTQYADRVFEHLNTEDELVLIHDYHLFALPTILRHKSPRARIGFFLHSPFPPTSE

ZmTPSII.5.3/1-986 HSFLSANRAFADRLTEVLSPEDELVWIHDYHLLALPTFLRKRFPRAKVGFFLHSPFPSSSE

ZmTPSII.5.4/1-851 HSFLSANRAFADRLTEVLCPEDELVWIHDYHLLALPTFLRKRFPRAKVGFFLHSPFPSSSE

ZmTPSI.1.1_tps1/1-960 IYRTLPSRLELLRSVLCADLVGFHTYDYARHFVSACTRILGLEGTPE---GVEDQGRLT

ZmTPSI.1.2/1-576 -----

ZmTPSII.2.1/1-888 IYKTLPVREELLRSLNADLIGFHTFDYARHFVLSCCSRMLGLKYESQRYIALEYGRTV

ZmTPSII.2.2/1-863 IYKTLPVREELLRALLNSDLIGFHTFDYARHFVLSCCGRMLGLSYESKRGHICLEYGRTV

ZmTPSII.3.1/1-912 IYRTLPRDEILKALLNCDLIGFHTFDYARHFVLSCCSRMLGIEYQSKRGYIGLDYFGRTV

ZmTPSII.3.2/1-912 IYRTLPRDEILKALLNCDLIGFHTFDYARHFVLSCCSRMLGIEYQSKRGHIGLDYFGRTV

ZmTPSII.3.3/1-912 IYRTLPRDEILKALLNCDLIGFHTFDYARHFVLSCCSRMLGIEYQSKRGYIELDYFGRTV

ZmTPSII.4.1/1-864 IYRTLPRDEILKSLNADLIGFQTFDYARHFVLSCCSRLGLNYESKRGHIGIEYFGRTV

ZmTPSII.4.2/1-865 IYRTLPRDEILKSLNADLIGFQTFDYARHFVLSCCSRLGLHYESKRGYIGIEYFGRTV

ZmTPSII.4.3/1-868 IYRTLPRDEILKSLNADLIGFQTFDYARHFVLSCCSRLGLHYESKRGYIGIEYFGRTV

ZmTPSII.5.1/1-953 LFRTPVREDLLRSLLNADLVGFHNIDYARHFVLSACTRLLGVTSHTRGYISIDYCGRAV

ZmTPSII.5.2/1-860 LFRAPVREELLRALLNADLVGFQNYDYGCHFISACSTLLGITSRAHDYICIDYFGRAV

ZmTPSII.5.3/1-986 IFRTIPVRDDLVRALLNADLVGFHTFDYARHFVLSACSRLGLDYQSKRGYIGIEYGRTV

ZmTPSII.5.4/1-851 IFRTIPVRDDLVRALLNADLVGFHTFDYARHFVLTACSRLGLDYQSKRGYIGIEYGRTV

ZmTPSI.1.1_tps1/1-960 RVAAPFPIGIDSDRFKRALELPAVKRHVSELTERF--AGRKVMLGVDRLDMIKGIKIPQKILA

ZmTPSI.1.2/1-576 -----MLGVDRLDMIKGIKIPQKILA

ZmTPSII.2.1/1-888 TIKILPVGVHLEQLRSVNLNPELGVKVAELLKQFCHRNRLLLGLVDDMDIFKGISLKLLA

ZmTPSII.2.2/1-863 SIKILPVGVMHLEQLKTVLGLPETEAKVSELMEMYSKGRVVMGLVDDMDIFKGISLKLLA

ZmTPSII.3.1/1-912 GIKIMPVGVMHMQLESGLRLPDREWRLSELQQQF--QGKTVLLGVDDMDIFKGINLKLLA

ZmTPSII.3.2/1-912 GIKIMPVGVMHMQLESGLRLPDREWRLSELQQQF--QGKTVLLGVDDMDIFKGINLKLLA

ZmTPSII.3.3/1-912 GIKIMPVGVMHMQLELGLRLPDREWRLSELQRQF--QGKTVLLGVDDMDIFKGINLKLLA

ZmTPSII.4.1/1-864 SLKILAGVHVGRLEATLRLPATIKKVEIESRY--SGKLVILGVDDMDIFKGISLKLLG

ZmTPSII.4.2/1-865 SLKILSVGVHIGRLESVCLKLPATVSKVQIEQRY--KGKILMLGVDDMDIFKGISLKFLG

ZmTPSII.4.3/1-868 SLKILSVGVHVGRLESVCLKLPATVSKVEEIEQRY--KGKILMLGVDDMDIFKGISLKLLA

ZmTPSII.5.1/1-953 SVKILAGGVDIGQLREVLSPEEAKAKEVATK--AGRQLLLGVDDVDFKIGIGLKLLA

ZmTPSII.5.2/1-860 VVKILSVGVDVRLREVLSPEEAKAKEVATK--AGRQVLIGVDDVDFNRIDVKLLA

ZmTPSII.5.3/1-986 TVKILPVGIDMQLRVSVSAPETEDAVRRVTEAY--KGRRLMVGVDVDFKIGIGLKFLA

ZmTPSII.5.4/1-851 TVKILPVGIDMQLRVSVSAPETEDAVRRVTEAY--KGRRLMVGVDVDFKIGIGLKFLA

ZmTPSI.1.1_tps1/1-960 FEKFL EENPDWNNKVLLQIAVPT**R**TDVPEYQKLT SQVHEIVGRINGRFGTL-TAVPIHH
ZmTPSI.1.2/1-576 FEKFL EENPDWNDKVVLLQIAVPT**R**TDVPEYQKLT SQVHEIVGRINGRFGTL-TAVPIHH
ZmTPSII.2.1/1-888 FEQLLMQHPEWRGRVVLVQIANPA**R**GRGKDVREVQEESDAMVRRINDAFGQP-GYQPVIL
ZmTPSII.2.2/1-863 MEELLRQHPEWRGKLVLVQVANPA**R**GRGKDVAEVQETETYAMVRRINEVYGEP-GYEPVVL
ZmTPSII.3.1/1-912 FENMLRTHPKWQGRAVLVQIANPA**R**GRGKDLEAIQAEIEESCQRINVDGFGS-GYSPVVF
ZmTPSII.3.2/1-912 FENMLRTHPKWQGRAVLVQIANPA**R**GRGKDLEAIQAEIEESCQRINGDFGQS-GYSPVVF
ZmTPSII.3.3/1-912 FENMLRTHPKWQGRAVLVQIANPA**R**GRGKDLEAIQAEIEESCQRINGDFGQS-GYSPVVF
ZmTPSII.4.1/1-864 LELLLERTPKLRGKVVLVQIVNPA**R**SIGKDIEEAKYEASVAQRINDKYGSA-NYKPVVL
ZmTPSII.4.2/1-865 LELLLDRNPKLREKVVVLVQIINPA**R**STGKDVQEAIT EAVSVAERINTNYGSS-SYKPVVL
ZmTPSII.4.3/1-868 LELLLDRNPKLREKVVVLVQIINPA**R**STGKDVQEAIT EAVSVAERVNTKYGSS-SYKPVVL
ZmTPSII.5.1/1-953 MERLLESQPELHGQVVLVQINNPA**R**SPGYDTDEICAE LQAMKRINARFATPAGYEP IVI
ZmTPSII.5.2/1-860 MERLLESRPELIGQVVLVQINNPA**R**SPGRD TDTVLAEVQLLMDRINARFAKP-GYDPIVM
ZmTPSII.5.3/1-986 MEQLLVEHRELGRVVLVQIANPA**R**SEGRDVQGVQDEARAI SARVNARFGTP-GYTP IVL
ZmTPSII.5.4/1-851 MEQLLVEHRELGRHAVLVQIANPA**R**SEGRDVQGVQDEARAI SARVNARFGTP-GYTP IVL

ZmTPSI.1.1_tps1/1-960 LDRSLDFHALCALYAVTDVALVTS**LDGMNLV**SYEYVACQGS-----KKG
ZmTPSI.1.2/1-576 LDRSLDFHALCALYAVTDVALVTS**LDGMNLV**SYEYVACQGS-----KKG
ZmTPSII.2.1/1-888 IDQ**PL**QFYERMAYYVAECCLVAVR**DGMNL**IPY**EY**VIARQGNERIDSILGLGPASRKK S
ZmTPSII.2.2/1-863 IDE**PL**QFYERVAYYVIAEVCVAVR**DGMNL**IPY**EY**IVSRQGN EKLDRMLRQ GKPEKK S
ZmTPSII.3.1/1-912 INRDVSSVEKVA YYTIAECVVVAVR**DGMNL**TPY**EY**IVCRQGAPGSES VSEVSGP--KKS
ZmTPSII.3.2/1-912 IDRVDVSSVEKIA YYTIAECVVVAVR**DGMNL**TPY**EY**IVCRQGAPGSES VSEVSGP--KKS
ZmTPSII.3.3/1-912 IGRDVSSVEKIA YYTIAECVVVAVR**DGMNL**TPY**EY**VVCRQGAPG SSVSEVSGP--KKS
ZmTPSII.4.1/1-864 IDYSIPFYEKIAFYAASDCCIVNAVR**DGMNL**IPY**EY**TVCRQGN EELDKLRGLNKSSSHTS
ZmTPSII.4.2/1-865 IDHHIPFYEKIAFYAASDCCIVNAVR**DGMNL**VPY**EY**TVCRQGN EIDKLRGLGKDTHTS
ZmTPSII.4.3/1-868 IDNRIPFYEKVAFYAASDCCIVNAVR**DGMNL**VPY**EY**TVCRQGN EIDRVRGLDKDTHTS
ZmTPSII.5.1/1-953 IEDPMTMHEKLA FYTSADICLVAVR**DGLNR**TPYIYTVCRQEGPISSGVV GAP---KEG
ZmTPSII.5.2/1-860 IDDP**L**TMHEKLA FYTSADICIVAVR**DGLNR**TPYIYTVCREHGP ISSGVV GAP---RES
ZmTPSII.5.3/1-986 IDGPVTPQEKAAAYYAAAECCVLSAVR**DGLNR**IPYIYTVCRQES-----TALGDDAPKRS
ZmTPSII.5.4/1-851 IDAPVTPQEKAAAYYAAAECCVLSAVR**DGLNR**IPYIYTVCRQES-----TALGDDSPKRS

ZmTPSI.1.1_tps1/1-960 VLILSEFAGAAQSLGAGAILVNPWNITEVADSI RHALTMPSDEREKRHRHNYAHVTHTTA
ZmTPSI.1.2/1-576 VLILSEFAGAAQSLGAGAILVNPWNITEVADSI RHALTMPSDEREKRHRHNYAHVTHTTA
ZmTPSII.2.1/1-888 MLVVSEFIGCSPSLS-GAIRVNPWNIDS VADAMDYALEMPEGEKVL RHEKHRYVSTHDV
ZmTPSII.2.2/1-863 MLVVSEFIGCSPSLS-GAIRVNPWNIEAVADAMETALVLPEN EKRLRHDKHFVSTHDV
ZmTPSII.3.1/1-912 MLVVSEFIGCSPSLS-GAIRVNPWNIEATAEAMNEAISMPEQEKQLRHEKHRYVSSH DV
ZmTPSII.3.2/1-912 MLVVSEFIGCSPSLS-GAIRVNPWNIEATAEAMNEAISMPEQEKQLRHEKHRYVSSH DV
ZmTPSII.3.3/1-912 MLVVSEFIGCSPSLS-GAIRVNPWNIEATAEAMNEAISMPEQEKQLRHEKHRYVSSH DV
ZmTPSII.4.1/1-864 TLIVSEFVGCSPSLS-GAFRVNPWSMEDVADALYSVTDLTRY EKRLRHEKHRYVSSH DV
ZmTPSII.4.2/1-865 TLIVSEFVGCSPSLS-GAFRVNPWSVDDVADALCRATDLTSEKRLRHEKHRYVSTHDV
ZmTPSII.4.3/1-868 TLIVSEFVGCSPSLS-GAFRVNPWSVDDVADALCRATDLSESEKRLRHEKHRYVSTHDV
ZmTPSII.5.1/1-953 AIVLSEFVGCATSLG-GAVHINPWNVDAVAEGMHMALRFNGREKQVRQE KHRYFVSTHDI
ZmTPSII.5.2/1-860 AIVLSELVGCSTFLR-GAVRVNPWNVDDVVEGMSSALRLNERDKKILHAKHYMYVVKHDI
ZmTPSII.5.3/1-986 AIVLSEFVGCSPSLS-GAIRVNPWSVESVAEAMNAALRMPAEQRLRHEKHRYVSTHDV
ZmTPSII.5.4/1-851 VIVLSEFVGCSPSLS-GAIRVNPWSVESVAEAMNAALRMPAEQRLRHEKHRYVSTHDV

ZmTPSI.1.1_tps1/1-960 QDWAETFFVFLNDTVAEAL-----LRTRQVPPG---LPSQMAIQYLRSKNRL
ZmTPSI.1.2/1-576 QDWAETFFVFLNDTVAEAL-----LRTRQVPPG---LPGQTAIQYLRSKNRL
ZmTPSII.2.1/1-888 GYWANSFLQDLERICLDHNRRCWIGFGLKFRVVALDPNFKKLAVEHLVLAYRRTKRV
ZmTPSII.2.2/1-863 GYWANSFLDLERTCKYHSQKRCWIGFGLRFRVSLDLTFRKLSLENILMAYRRAKTRA
ZmTPSII.3.1/1-912 AYWSKSFILDLERACRDHFKRTCWIGLGFGRVVALDPHFRLNMDSIVNAYEISESRA
ZmTPSII.3.2/1-912 AYWSKSFILDLERACRDHFKRTCWIGLGFGRVVALDAHFRKLNMDSIVNAYEISGSRA
ZmTPSII.3.3/1-912 AYWSKSFIDLERVCKDHFKRTCWIGLGFGRVVALDPHFRTKLNMDSIINAYEISESRA
ZmTPSII.4.1/1-864 AYWARSFQDLKACIEQYSQRCWTTGFGLNFRVIALSPGFRRLSLEHLASSYKKANRRM
ZmTPSII.4.2/1-865 AYWARSFQDLERACKDHYSRRCWAIGFGLNFRVIALSPGFRKLSSEHFVSSYNKASRRA
ZmTPSII.4.3/1-868 AYWAHSFAQDLERACRDHYSRRCWAIGFGLNFRVIALSPGFRKLSSEHFVSSYNRASRRA
ZmTPSII.5.1/1-953 AYWGRSLDQLQRASKDHASMKFMSVGLAMSYHIVVLSPNFQKLSPEHINPSYQRAGNRL
ZmTPSII.5.2/1-860 AYWGRSLDQNLQKASMDHASMNFLSVGLAMNFRIVVLDPNFQKLSPEHINPSYHRTGNRL
ZmTPSII.5.3/1-986 AYWARSFSDQLQRACKDHFSTRHWIGFMSFKVVALGPNFRRLSVEHIVPSYRRTENRL
ZmTPSII.5.4/1-851 AYWARSFSDQLQRACKDHFSTRHWIGFMSFKVVALGPNFRRLSVEHIVPSYRRTDNRL

MOTIF I

MOTIF II

ZmTPSI.1.1_tps1/1-960 LILGFNS**TL**TEPVESSGRRGGDIKEMELKLPDLKGPLRALCEDERTTVIVL**SGS**DRSV
ZmTPSI.1.2/1-576 LILGFNS**TL**TEPVESSGRRGGDIKEMELKLPDLKGPLGALCEDERTTVIVL**SGS**DRSV
ZmTPSII.2.1/1-888 ILL**DYD**GTLM-PQ**T**SLGKSPTSRTIDM-----LNSLCRDRNMVFLV**SAKSRMT**
ZmTPSII.2.2/1-863 ILL**DYD**GTLM-PQ-AINKSPSTESVRI-----LNSLCRDKDNVVYLC**SGYDRRT**
ZmTPSII.3.1/1-912 ILL**DYD**GTLV-PQ**T**SINKEPSPEVLNI-----INTLCSDSRNIVFLV**SGRDKDT**
ZmTPSII.3.2/1-912 ILL**DYD**GTLV-PQ**T**SINKEPSPEVLNI-----INTLCSDSRNIVFLV**SGRDKDM**
ZmTPSII.3.3/1-912 ILL**DYD**GTLV-PQ**T**SLNKEPSPQVLSI-----INTLCSDSRNIVFLV**SGRDKDT**
ZmTPSII.4.1/1-864 I**FLDYD**GTLV-PQ**T**SHDKSPSAELIST-----LNSLCSMDKNTVFIV**SGRGRDS**
ZmTPSII.4.2/1-865 I**FLDYD**GTLV-PQ**S**SINKAPSEEVISV-----LNTLCNDPKNIVFIV**SGRGRDS**
ZmTPSII.4.3/1-868 I**FLDYD**GTLV-PQ**S**SINKAPSEEVISI-----LNTLCNDPKNVFIV**SGRGRDS**
ZmTPSII.5.1/1-953 ILL**DYD**ETVMFHPGLLDRHPSQRLIGI-----LNELCSDPKNTVFVV**SGRSKDE**
ZmTPSII.5.2/1-860 ILL**DYD**GTVMV**P**QGLITRHPSEQELVSV-----LNELCSDPMNTVFVV**SGRSKDE**
ZmTPSII.5.3/1-986 ILL**DYD**GTVM-PENSIDRTPSSEVISV-----LNRLCEDPKNRVFIV**SGRGKDE**
ZmTPSII.5.4/1-851 ILL**DYD**GTVM-PENSIDRTPSSEVISV-----LNRLCEDPKNRVFIV**SGRGKDE**

ZmTPSI.1.1_tps1/1-960 LDENFGEF-KMWLAAEHGMFLR-PTYGEWMTTMEHLNMDWVDSVKHVFEYFTERTPRSH
ZmTPSI.1.2/1-576 LDENFGEF-KMWLAAEHGMFLR-PTYGEWMTTMEHLNMDWVDSVKHVFEYFTERTPRSH
ZmTPSII.2.1/1-888 LNEWFLPCESLGLAAEHGCFRLRLRDAEWETCVPV-IDCSWKQIAEPVMKTYTETTDGST
ZmTPSII.2.2/1-863 LHEWF-PCENLGLIAAEHGYFLRCKRDAEWKTCVAA-TDCSWKQIAEPVMCLYRETDDGST
ZmTPSII.3.1/1-912 LGKWFSSCPKLGIAAEHGYILRWSSEEWQCTQA-MDFGWMQMAKPVMNLYTEATDGSY
ZmTPSII.3.2/1-912 LGKWFSSCPKLGIAAEHGYFLRWSSEEWQCTQA-MDFGWMQMAKPVMNLYTEATDGSY
ZmTPSII.3.3/1-912 LGKWFSSCPRLGIAAEHGYFLRWSREEEWQCTQA-LDFGWMQMAKPVMNLYTEATDGSY
ZmTPSII.4.1/1-864 LSEWFASCENLGLIAAEHGYFIRWNKAAEWETSFSG-IYSEWKLADPIMHVYMETDDGSF
ZmTPSII.4.2/1-865 LDEWFSPEKLGIAAEHGYFIRWSKEAAWESSYSR-PQEWKHIAEPVMQVYTTETDGSY
ZmTPSII.4.3/1-868 LDEWFSPEKLRLLAAEHGYFIRWSKEAAWESSYSS-PRQEWKHIAEPVMQVYTTETDGSY
ZmTPSII.5.1/1-953 LARWLEPCEERLGLIAAEHGYFTRWSRYPWESPDLK-VDYGWKMKVEFVMDLYAVTDGSS
ZmTPSII.5.2/1-860 LAGWLAPCEKLGIAAEHGYFTRWSRDPWESPDKLL-LDNDWKNIVEPVMKYCDVTDGSY
ZmTPSII.5.3/1-986 LSRWFAPCEKLGIAAEHGYFTRWSRDAPWEASALA-ADLDWKNIAEPVMRLYTEATDGSY
ZmTPSII.5.4/1-851 LSRWFAPCEKLGIAAEHGYLTRWSRDAPWDTSGLA-ADFDWKKTAEPVMQLYTEATDGSY

ZmTPSI.1.1_tps1/1-960 FEHRETSFVWNYKYADVEFGRLQARDMLQHLWTGPI SNAAVDVVQGSRSVEV--RSVG-V
ZmTPSI.1.2/1-576 FEHRETSFVWNYKYADVEFGRLQARDMLQHLWTGPI SNAAVDVVQGSRSVEV--RSVG-V
ZmTPSII.2.1/1-888 IEDKETAIVWCYEDADPDFGSCQAKELHDHL-ESVLANEPVSVKAGPNLVEV--KPGQ-V

ZmTPSII.2.2/1-863 IEDRETVLVWNYEDADPDFGSSQAKELVDHL-ESVLANEVPSVKTPHSVEV--KPQG-V
 ZmTPSII.3.1/1-912 IERKESALVWHHQDADPDFGSSQAKELLDHL-ESVLANEVPSVKSGQFIVEV--KPQG-V
 ZmTPSII.3.2/1-912 IETKESALVWHHQDADPDFGSSQAKELLDHL-ESVLANEVPSVKSGQFIVEV--KPQG-V
 ZmTPSII.3.3/1-912 IEAKESALVWHHQDADLGFSSQAKEMLDHL-ESVLANEVPSVKSGQFIVEV--KPQG-I
 ZmTPSII.4.1/1-864 IEPKESALVWHYQNTDHDGFGSCQAKELVSHL-ERVLSNEPVVRRGHQIVEV--KPQG-V
 ZmTPSII.4.2/1-865 IESKESALVWHYLDADHDGFGSFQAKELQGH-ERVLSNEPVVVKCGHYIVEV--KPQG-V
 ZmTPSII.4.3/1-868 VESKESALVWHYLDADHDGFGSFQAKELKDHL-ERVLSNEPVVVKCGHYIVEV--KPQG-V
 ZmTPSII.5.1/1-953 VETKETALVWHYEGTDPVFGPSQAKELRDHL-SDVLAKEPVSVRSYNIIVEV--NPQE-V
 ZmTPSII.5.2/1-860 IEAKETALVWHYEEADPDFGPRQAKELQYHL-RDVLSEEPVYKSGHQIVEVNGNPQEVV
 ZmTPSII.5.3/1-986 IEHKESGMVWHHDEADPDFGSCQAKELLDHL-ENVLANEVSVKRGQHIVEV--NPQG-I
 ZmTPSII.5.4/1-851 IEHKESAIVWHHDEADPDFGSCQAKELLDHL-ENVLANEVSVKRGQHIVEV--NPQG-I

MOTIF III

ZmTPSI.1.1_tps1/1-960 TKGAAIDRILGEIV-HSENMITP-IDYVLCIGHFLGKDEDIYVFFDPEYPSSESKVKPEGG
 ZmTPSI.1.2/1-576 TKGAAIDRILGEIV-HSENMITP-IDYVLCIGHFLGKDEDIYVFFDPEYPSSESKVKPEGG
 ZmTPSII.2.1/1-888 SKGLVAKRILSTTQ-ERGDADDDLPDFVLCVGD-DRSDEDMF-----EVIAAAAA-
 ZmTPSII.2.2/1-863 SKGLVARRMLVSMK-ERGCQ---PDFVLCIGD-DKSDDEDMF-----QLIATAAC-
 ZmTPSII.3.1/1-912 SKGIVAERILASVK-ERGCQ---ADFLCIGD-DRSDEDMF-----ENIADI IK-
 ZmTPSII.3.2/1-912 SKGIVAERILASVK-ERGCQ---ADFLCIGD-DRSDEDMF-----ENIADI IK-
 ZmTPSII.3.3/1-912 SKGIVAERILASVK-ERGCQ---ADFLCIGD-DRSDEDMF-----ENIADI IG-
 ZmTPSII.4.1/1-864 SKGISVDKIIRTLV-SKGEV---PDLMLCIGN-DRSDEDMF-----ESINRATS-
 ZmTPSII.4.2/1-865 SKGLAVNKLIIHTLV-KNGKA---PDFLMCVGN-DRSDEDMF-----ESINGMTS-
 ZmTPSII.4.3/1-868 SKGRAVDKLIQALANNNGKA---QDFLMCVGN-DRSDEDMF-----ECINGMAS-
 ZmTPSII.5.1/1-953 DKGTAVQRIIAAMR-DRGRM---PDFILCVGD-DASDEDMF-----KAVTAPSN-
 ZmTPSII.5.2/1-860 GKGTAVQGLIAALG-ARGRM---PDFILCVGD-DVSDDEDMF-----EAISAPSS-
 ZmTPSII.5.3/1-986 SKGVVVDLSSSMV-RTGKP---PDFVLCIGD-DRSDEDMF-----ESIVCPAS-
 ZmTPSII.5.4/1-851 SKGVVVDLSSSMV-RTGKP---PDFVLCIGD-DRSDEDMF-----ESIVCPAS-

MOTIF III

ZmTPSI.1.1_tps1/1-960 SASLDRRPNGRPPSNGRSNSRNPQSRTPQKAQQAASERSSSSSSSTSSNHDWREGSSVLD
 ZmTPSI.1.2/1-576 SASLDRRPNGRPA SNGRSNSRNPQSRPQKAQQAASERSSSSSSSTSSNHDWREGSSVLD
 ZmTPSII.2.1/1-888 -----ARGVSSLQ
 ZmTPSII.2.2/1-863 -----G---DSLA
 ZmTPSII.3.1/1-912 -----R---NMVA
 ZmTPSII.3.2/1-912 -----R---NMVA
 ZmTPSII.3.3/1-912 -----R---NLVA
 ZmTPSII.4.1/1-864 -----LSELP
 ZmTPSII.4.2/1-865 -----N---AVLS
 ZmTPSII.4.3/1-868 -----N---DVSS
 ZmTPSII.5.1/1-953 -----K---SAFP
 ZmTPSII.5.2/1-860 -----KF---AFP
 ZmTPSII.5.3/1-986 -----SSGGVRLP
 ZmTPSII.5.4/1-851 -----NSG-VKLP

ZmTPSI.1.1_tps1/1-960 LKGENYFSCAVGRKRSNARYLLSSSEEVVSFLKELATA-----TA
 ZmTPSI.1.2/1-576 LKAENYFSCAVGRKRSNARYLLSSSEEVVSFLKELATE-----TA
 ZmTPSII.2.1/1-888 AEA-EVFACTVGRKPSKAKYYLDDPADIVRLVQGLASV-----SDDQTHAPPPPPP
 ZmTPSII.2.2/1-863 SKA-EVFACTVGRKPSKAKYYLDDAAEVVRLMQGLSYV-----SEELANQRD

ZmTPSII.3.1/1-912 PRT-SLFACTVGGQKPSKAKFYLDLDTF EVVAMLSALADATGAELKSDSADELAASISSLDI
ZmTPSII.3.2/1-912 PRT-SLFACTVGGQKPSKAKFYLDLDTF EVVAMLSALADATGAELKSDSADELAASISSLDI
ZmTPSII.3.3/1-912 PRT-ALFACTVGGQKPSKAKFYLDLDTF EVVTMLSALADATGPELETDSADEVAYISSLDI
ZmTPSII.4.1/1-864 AAP-EVFACSVGPKASKANYVDGCE EVIRLLKGVTA-----SLQKDTA
ZmTPSII.4.2/1-865 PTMPELFACTVGGQKPSKAKFYLDLDTF EVIRLLKKNVTRI-----PSQRQDVSA
ZmTPSII.4.3/1-868 TTVPEVFACSVGQKPSKAKFYLDLDTF EVIRLLRDATRF-----SSSQREDVNA
ZmTPSII.5.1/1-953 EDA-EVFACTIGTKPSLAKYYLDLDPV EVLSMLKGLIKS-----SVEERP
ZmTPSII.5.2/1-860 EAA-EIFACTVGNKPSLAKYYLEDPD EVLKMLKGLIDS-----FEE
ZmTPSII.5.3/1-986 ASS-EVFACTVGGKPSMAKYYLDLDTV DVVKMLDGLASA-----PSPRRPG
ZmTPSII.5.4/1-851 ASS-EVFACTVGGKPSMARYLDLDTV DVVKMLDGLASA-----PSQQR

ZmTPSI.1.1_tps1/1-960 GFQATCADYMHVLG-
ZmTPSI.1.2/1-576 GFQSSCADYMFLLDRQ
ZmTPSII.2.1/1-888 SAATTAADTVPR---
ZmTPSII.2.2/1-863 EDEDSSLDDVWE---
ZmTPSII.3.1/1-912 GDEQSESDTPIGGS
ZmTPSII.3.2/1-912 GDEQSETSDTPIGGS
ZmTPSII.3.3/1-912 GDEQSESSDKPVEGS
ZmTPSII.4.1/1-864 GHSHAAFEDTLEVVS
ZmTPSII.4.2/1-865 SHGRVTFRGVLDYVD
ZmTPSII.4.3/1-868 SRGRVTFRDALDYVD
ZmTPSII.5.1/1-953 GDGEGPSRVSFD---
ZmTPSII.5.2/1-860 NHSTVEANK-----
ZmTPSII.5.3/1-986 PAAAVQLRVSFEGSL
ZmTPSII.5.4/1-851 SRPAVQLRVSFEGSL

Supplemental Figure S2B. Predicted enzymatic domains for TPP proteins.

TPP predicted domain; Conserved motifs **HAD phosphatases**

ZmTPPA.1/1-388 --MDLKTGLNS---PVIADHLPTLALPAA-VMTFTTPTSFP---SP-----
ZmTPPA.3/1-369 --MDMGSG-SS---PVITDPISISPLLGLTSLNLMFVSVM---SG-----
ZmTPPB.1.1/1-367 --MTKQGMVVPV--PEAAVAVPPNSAP---LFQYPPRAAP---GV-----
ZmTPPB.1.2/1-384 MPMAKPSVAEASGVPAQASCSCPCPGTTLFPYPPPRGASGIAAA-----
ZmTPPB.1.3/1-370 --MAKPSVAVPEV-GVPAAQA-SCTCPGT-LLAYPPRGAG--VAA-----
ZmTPPB.1.4/1-357 -MTNQDQVVVSEM-GIAAGAALPGPSPA--LLAC---RGAA---AG-----
ZmTPPB.1.5/1-356 --MTNQDQVVVSEM-GIAAGTALPGSSPA--LLAC---RGAA---AG-----
ZmTPPB.1.6/1-384 --MTNQDQVVVSDM-GIAAAAAALP--PPG--LFACRGVAGAVSSLRGTYSGLGLPGGAAAD
ZmTPPB.2.1_ramosa3/1-361 --MTKHAAYSSED-VVAVAAPA--PAGRHTSFQALKGAP---L-----
ZmTPPB.2.2/1-327 --MTKHTAFAGADGGTTAAAA-----VTLCAPRA----RG-----
ZmTPPB.2.3/1-357 --MTKRFAFAADD-AIIAAAAVTSQPGRRTSYPPARA----RG-----

ZmTPPA.1/1-388 GLCLNTTKKIPLPGKIEEVRAAG-W-LDLMKASSPTRKRQIKDVICDAQSD---LDLQYC
ZmTPPA.3/1-369 GCSSSPSMSASSRRKIEEVLVNG-L-LDAMKSSSPRKKHNL-AFGQDNSPD---EDPAYT
ZmTPPB.1.1/1-367 AVRKKCLQMGAGAGRI-----GGW-VESMRASSPTHAKAAAALAGV-----EEERYA
ZmTPPB.1.2/1-384 AVRRKCLQAEVGGGAC-----WGVESMRASSPTHARAAAALAGAGAD---EEEERA
ZmTPPB.1.3/1-370 AVRRKCLQVELGAGAG-LLGGAGAWGVESMRASSPTHARAAAALAGGGVDVDVDEERA
ZmTPPB.1.4/1-357 AMSLRYLDLAAAAARS---ASGTW-ADAMRASSPTRSRAA-----DEFT
ZmTPPB.1.5/1-356 AMSLRYLDLAAAAARS---ASCTW-VEAMRASSPTRSRAAADV-----DELT
ZmTPPB.1.6/1-384 GGEFRSPVAAAANAPPGRSCTSRV-VEAIRASSPARCPAV-----DEYD
ZmTPPB.2.1_ramosa3/1-361 DCKKHAVDLSASGAAVV--GGGPW-FESMKASSPRRAADA-----EHG
ZmTPPB.2.2/1-327 -----ARRV---AAGSL-PELVRRHA-----DLD
ZmTPPB.2.3/1-357 GCRLAPAVAAAAARQATDDPGAAGSW-PELV---VPRHA-----DFD

MOTIF I

ZmTPPA.1/1-388 NWTVNYPALISFEAISDLGSKRLALFLDYDGTLSPIVDNPNALMSDEMRAAVRHAAS
ZmTPPA.3/1-369 AWLSKCPALASFKQIVANAQGRRIAVFLDYDGTLSPIVDDDPKAFMSPVMRAAVRNVAK
ZmTPPB.1.1/1-367 AWMVKHPSALAMFDQLVAASKGKQIVVFLDYDGTLSPIVDDPDAAAYMSDMRRAVRSVAK
ZmTPPB.1.2/1-384 AWMARHPSALGKFERIVAASEGRRIVMFLDYDGTLSPIVDDPDAAFMSETMRMAVRSVAK
ZmTPPB.1.3/1-370 SWMARHPSALGRFERIVAAEGRKIVMFLDYDGTLSPIVDDPDAAFMSETMRMAVRSVAK
ZmTPPB.1.4/1-357 AWRKHPSALGKFEQIASASKGKVVVMFLDYDGTLSPIVADPDAAAYMSDAMRAAVRDVAK
ZmTPPB.1.5/1-356 AWMRKHPSALGKFEQIASASQGGKVVVMFLDYDGTLSPIVADPDAAAYMSDVMRAAVRDVAK
ZmTPPB.1.6/1-384 AWTRKHPSALGSFDQIAAAAKGKRVVMFMDYDGTLSPIVADPDMAFMTPEMRAAVRNVAK
ZmTPPB.2.1_ramosa3/1-361 DWMEKHPSALAQFEPLLAAAKGKQIVMFLDYDGTLSPIVEDDPDRAVMSEEMREAVRRVAE
ZmTPPB.2.2/1-327 DWMEKHPSALAGFESVLAEEGKQVVMFLDYDGTLSPIVKDPDSAVMSEEMRDVAVRVAE
ZmTPPB.2.3/1-357 DWMEKHPSALAAFESVLAEEGKQIVMFLDYDGTLSPIVRDPDSAVMSEEMRDVAVRVAE

MOTIF II

ZmTPPA.1/1-388 LFPTAII SGRSRDKVDFVFKLNELYYAGSHGMDIMGP--VRKTTDSNGVEICIRSTDVHGK
ZmTPPA.3/1-369 YFPTAIV SGRSRKKVFEFVKLTELYYAGSHGMDIVTS-----AAAHATEKC-----K
ZmTPPB.1.1/1-367 HFPTAIV SGRCRDKVFEFVKLAELYYAGSHGMDIKGP---AKGSRHT-KA-----KG
ZmTPPB.1.2/1-384 HFPTAIV SGRCRDKVGFVVKLAELYYAGSHGMDIKGP--AKASSRHE-KA-----KA
ZmTPPB.1.3/1-370 HFPTAIV SGRCRDKVFEFVKLAELYYAGSHGMDIKGPAAAKASSSRH-AA-----KA
ZmTPPB.1.4/1-357 HFPTSIV SGRCRDKVRNFVLSLELYYAGSHGMDIKGP-----SS-----NP
ZmTPPB.1.5/1-356 HFPTAIV SGRCRDKVRSFVLDLSELYYAGSHGMDIEGP-----SS-----NP

ZmTPPB.1.6/1-384 RFPTAIV**TGR**CIEKVCFSVGLPELYYAGSHGMDIKGP-----NS-KE-----DK
ZmTPPB.2.1_ramosa3/1-361 HFPTAIV**SGR**CRDKVLNFVKLTELYYAGSHGMDIQGP--AACRQPNHVQQA-----EA
ZmTPPB.2.2/1-327 HFPTAIV**SGR**CRDKVFNFKLAELYAGSHGMDIKGP----TAQSKHT-KA-----KA
ZmTPPB.2.3/1-357 HFPTAIV**SGR**CRDKVFNFKLAELYAGSHGMDIKGP----TAQSKHT-KA-----KA

ZmTPPA.1/1-388 EVNLFQP--ASEFLPMITEVYEKLGESVKDIDGARMEDNKFCVSVHYRNVAEDDYKVKVFH
ZmTPPA.3/1-369 EANLFQP--ACEFLPMINEVSKCLVEVTSSIEGARVENNKFCVSVHYRNVAEKDWKVVAG
ZmTPPB.1.1/1-367 GGVLFPQ--ASQFLPMIEQVHDSLVEKTKCIPGAKVENNKFCVSVHFRCVDEKSWITLAD
ZmTPPB.1.2/1-384 KGVLFQPATASEFLPMIEAVHERLVETTRSIPGAKVENNRFCVSVHFRCVDEKMWGELWE
ZmTPPB.1.3/1-370 KGVVFPQ--ASEFLPMIEEVHERLVQTRCIPGAKVENNRFCVSVHFRRVDEKMWGELSE
ZmTPPB.1.4/1-357 ESVLCQP--ASEFLPMDEVYKALVEKTKSTPGAKVEHNKFCVSVHFRCVDEKRWGLAE
ZmTPPB.1.5/1-356 ESVLCQP--ASEFLPVIDEVYKALVEKTKSTPGAKVENNKFCVSVHFRCVDEKRWALAE
ZmTPPB.1.6/1-384 TVLLLPQ--AREFLPVIDKAYKALVEKTKDITGARVENNKFCVSVHFRCVDEKSWSSLAE
ZmTPPB.2.1_ramosa3/1-361 AAVHYQA--ASEFLPVIEEVFRTLAKMESIAGARVEHNKYCLSVHFRCVREEEWNVA
ZmTPPB.2.2/1-327 EAVLCQP--ASAFLPVIDEAYRALTARTAPIPGATVENNKFCVSVHFRCVQEEKWRALEE
ZmTPPB.2.3/1-357 GAVLCQP--ARAFLPVIEEVYRALTASTAPIPGATVENNKFCVSVHFRCVQEEKWRALEE

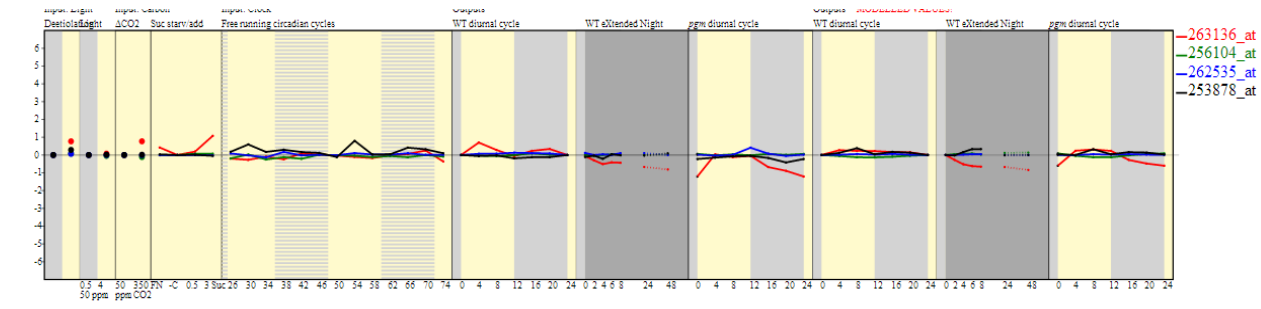
MOTIF III

ZmTPPA.1/1-388 RVTAVLEGYPCRLRLTHGRKVFVVRPVIDW**NK**GKAVEFLLESLGL-SESEDVLPVIYV**GDDR**
ZmTPPA.3/1-369 LVKQVLEAFPRRLKVTNGRMVLEVRPVIDW**DK**GKAVEFLRLSLGL-SDSEVVPIYI**GDDR**
ZmTPPB.1.1/1-367 MVKSVLKDYPKLKLTKQGRMVFEVRPTIKW**DK**GKALEFLLESLGY-ADCTDVLVPVI**GDDR**
ZmTPPB.1.2/1-384 SVKGVLRREYPRRLTKQGRMVLEVRPTIKW**DK**GKALEFLLESLGF-AGCTNVLPVYI**GDDR**
ZmTPPB.1.3/1-370 SVRGVLRGYPKLRLTHGRMVLEVRPSIKW**DK**GKALEFLLESLGF-ADCSSVLPVYI**GDDR**
ZmTPPB.1.4/1-357 QVKAVIDKYPKLKLTHGRKVLIRPSIMW**DK**GKALEFLLESLGF-ANRSDVLPVYI**GDDR**
ZmTPPB.1.5/1-356 QVKAVIDKYPKLKLTKQGRKVLIRPSIMW**DK**GKALEFLLESLGF-ASCSDALPVYI**GDDR**
ZmTPPB.1.6/1-384 KVKAVLRDFPELELTEGRKVVVVRPSIMW**DK**GKAVEFLRLSLGFDDRTNVLPVYI**GDDR**
ZmTPPB.2.1_ramosa3/1-361 EVRSVLRREYPNLKLTHGRKVLIRPSIKW**DK**GKALEFLLESLGY-AGRNDVFPVIYI**GDDR**
ZmTPPB.2.2/1-327 QVRSVLRKEYPDLRLTKGRKVLIRPSIKW**DK**GNAIQFLLECLGF-ADSNNVFPVIYI**GDDR**
ZmTPPB.2.3/1-357 QVRSVLRKEYPDLRLTKGRKVLIRPSIKW**DK**GNALQFLLESLGF-AGSNSVFPVIYI**GDDS**

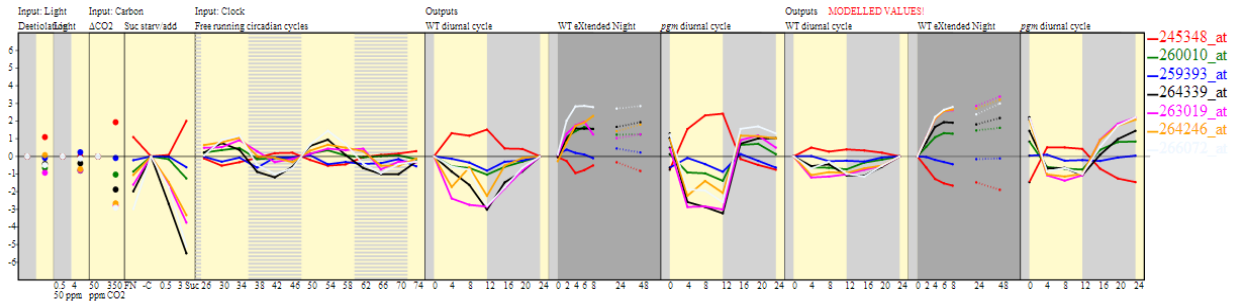
ZmTPPA.1/1-388 **TDEDAFKVLKASN**--RFGGILVSSIPKESDAFYSLRDP**AEV**THSTVATTNFCFEYFLGQ
ZmTPPA.3/1-369 **TDEDAFKVLRERS**--CGYGILVSQVPKDTEAFYSLRDP**SEVMG**-----FLNSLVR
ZmTPPB.1.1/1-367 **TDEDAFKVLRKRG**--QGVGILVSKHKPDTCASYS**LQEP**AEVME-----FLLRLVE
ZmTPPB.1.2/1-384 **TDEDAFRALRRRGQDQGVGILVSKHPKETSASYS**LQEP**AEVME**-----FLLRLVE
ZmTPPB.1.3/1-370 **TDEDAFKVLRRRGQDQGVGILVSKHPKETSASYS**LQGP**AEVRA**-----
ZmTPPB.1.4/1-357 **TDEDAFKVLRKRG**--QGIGILVSKCPKETNASYS**LQDP**GEVMD-----FLLRLVD
ZmTPPB.1.5/1-356 **TDEDAFKVLRKRG**--QGVGILVSKCPKETNASYS**LQDP**GEVMD-----FLLRLVE
ZmTPPB.1.6/1-384 **TDEDAFKVLRERG**--QGIGILVSKCPKETDATYS**LQDP**TEVME-----FLVRLGQ
ZmTPPB.2.1_ramosa3/1-361 **TDEDAFKVLRNMG**--QGIGILVSKLPKETAASYS**SLDP**AEVKE-----FLRKLAN
ZmTPPB.2.2/1-327 **TDEDAFKVLRGMG**--QGIGILVSKIPKETSASYS**SLR**EPS**EVKE**-----FLHMLVR
ZmTPPB.2.3/1-357 **TDEDAFKVLRNLG**--QGIGILVSKIPKETRASYS**SLR**EPS**EVVE**-----FLRKLVS

ZmTPPA.1/1-388 KEN-----
ZmTPPA.3/1-369 WKKHPL-----
ZmTPPB.1.1/1-367 WERLSKARPKW-----
ZmTPPB.1.2/1-384 WKRLSRLSRTQ-----

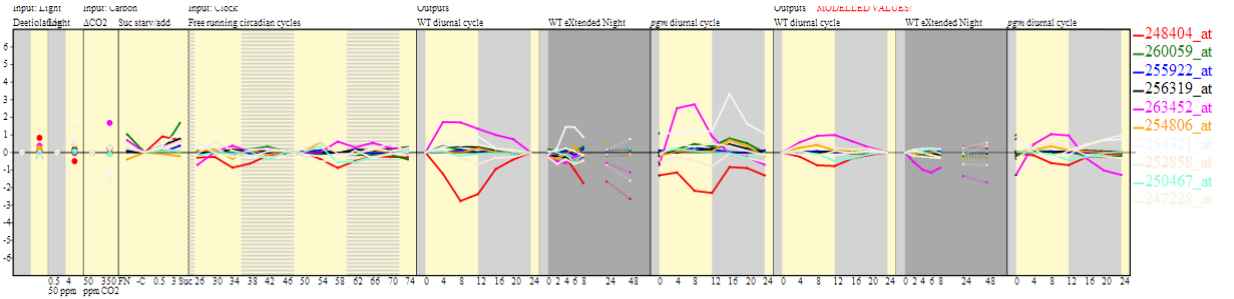
ZmTPPB.1.3/1-370	-----
ZmTPPB.1.4/1-357	WKRKSSAAPMVRPRV---
ZmTPPB.1.5/1-356	WKRKSTTRPPV-----
ZmTPPB.1.6/1-384	WNPLRSPSPAARPRGRKQ
ZmTPPB.2.1_ramosa3/1-361	KKGARQP-----
ZmTPPB.2.2/1-327	SKQR-----
ZmTPPB.2.3/1-357	WSKESRQRD-----



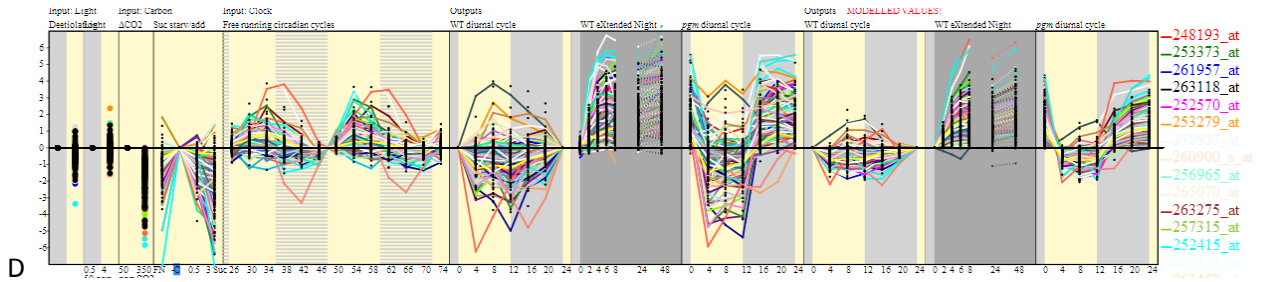
A



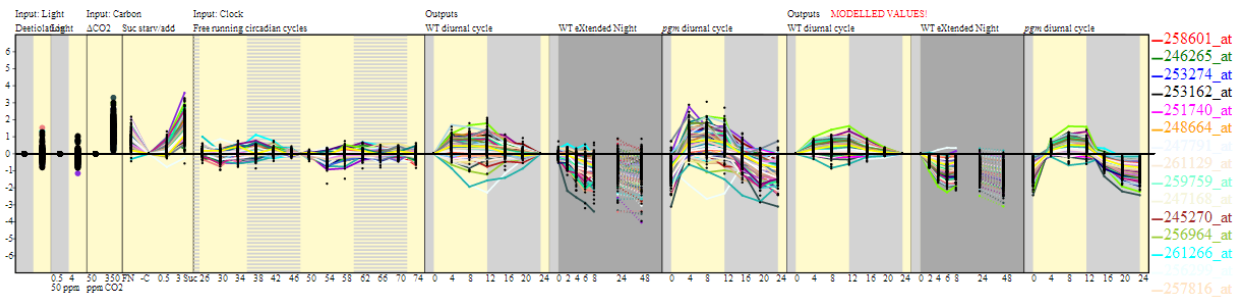
B



C



D



E

Supplemental Figure S3. Expression of Arabidopsis (A) class I TPS genes, (B) class II TPS genes, (C) TPP genes, (D) SNRK1 (inducible) targets, (E) SNRK1 (repressible) targets in mature leaf tissue throughout the diurnal cycle after 4 h extended night.

SUPPLEMENTARY DATA

Supplemental Methods S1. Methods for sugars and starch analysis.

Supplemental Method S2. Formulae for sugar and starch (glucose) analysis.

Supplemental Table T1. Gene names and accession numbers for maize, rice, Arabidopsis, and poplar TPS and TPP.

Supplemental Table T2. Sequence of primers used for qPCR.

Carbohydrate Metabolite Analysis

Frozen tissues (20-100 mg) were weighed and ground for 30-60 s while frozen using a Tissue Lyser II (Qiagen). Sugars (sucrose, hexoses and starch) were then extracted following a method adapted from Lunn et al. 2006. Tissues were resuspended in 500 μ L of ice cold $\text{CHCl}_3/\text{CH}_3\text{OH}$ (3:7, v/v). 250 nmols of lactose was added as an internal standard to calculate recovery. Soluble sugars were extracted for 2 h at -10°C with shaking in an orbital mixer (Model 5C25, Cole Parmer) for 5 min every 15 min interval, followed by two extractions with 400 and 200 μ L of water. Samples were then vortexed, incubated for 5 min at 4°C , centrifuged for 5 min at 10,000g and 200 μ L of the upper aqueous phase was collected and dried using a centrifugal vacuum. The residue was resuspended in 250 μ L of water and filtered by centrifugation for 2 h at 2250g using a MultiScreen® Ultracel-10 filter plates (Millipore) with samples covered with mineral oil to prevent evaporation. The filtrate was used directly for sugars analysis as described below. Starch was extracted from the pellet generated while extracting soluble sugars. The remaining upper phase was removed and the pellet was washed with 1 mL ice cold CH_3OH . The supernatant was removed and the pellet was dried using a centrifugal vacuum. The dried material was resuspended with 200 μ L 0.5 M NaOH and incubated for 1 h at 60°C with shaking to dissolve the starch. The solution was neutralized with 200 μ L of 0.5 M HCl, and the starch digested in 1 U amyloglucosidase (Roche Diagnostics), 600 μ L 0.2 M NaOAc (pH 4.5) and 1 μ mol lactose as internal standard for 12 to 24 h at 30°C with shaking. The reaction was stopped by boiling samples for 2 min. Samples were then centrifuged to remove debris and the supernatant was transferred to a new microcentrifuge tube. 250 μ L of digested starch was filtered as described previously for soluble sugars. Samples were diluted 1:100 in filtered ultrapure water for soluble sugars and 1:10 for hydrolyzed starch.

Carbohydrate Metabolite Analysis

Frozen tissues (20-100 mg) were weighed and ground for 30-60 s while frozen using a Tissue Lyser II (Qiagen). Sugars (sucrose, hexoses and starch) were then extracted following a method adapted from Lunn et al. 2006. Tissues were resuspended in 500 μ L of ice cold $\text{CHCl}_3/\text{CH}_3\text{OH}$ (3:7, v/v). 250 nmols of lactose was added as an internal standard to calculate recovery. Soluble sugars were extracted for 2 h at -10°C with shaking in an orbital mixer (Model 5C25, Cole Parmer) for 5 min every 15 min interval, followed by two extractions with 400 and 200 μ L of water. Samples were then vortexed, incubated for 5 min at 4°C , centrifuged for 5 min at 10,000g and 200 μ L of the upper aqueous phase was collected and dried using a centrifugal vacuum. The residue was resuspended in 250 μ L of water and filtered by centrifugation for 2 h at 2250g using a MultiScreen® Ultracel-10 filter plates (Millipore) with samples covered with mineral oil to prevent evaporation. The filtrate was used directly for sugars analysis as described below. Starch was extracted from the pellet generated while extracting soluble sugars. The remaining upper phase was removed and the pellet was washed with 1 mL ice cold CH_3OH . The supernatant was removed and the pellet was dried using a centrifugal vacuum. The dried material was resuspended with 200 μ L 0.5 M NaOH and incubated for 1 h at 60°C with shaking to dissolve the starch. The solution was neutralized with 200 μ L of 0.5 M HCl, and the starch digested in 1 U amyloglucosidase (Roche Diagnostics), 600 μ L 0.2 M NaOAc (pH 4.5) and 1 μ mol lactose as internal standard for 12 to 24 h at 30°C with shaking. The reaction was stopped by boiling samples for 2 min. Samples were then centrifuged to remove debris and the supernatant was transferred to a new microcentrifuge tube. 250 μ L of digested starch was filtered as described previously for soluble sugars. Samples were diluted 1:100 in filtered ultrapure water for soluble sugars and 1:10 for hydrolyzed starch.

Sugar peaks were identified in comparison with known sugars and following formulas:

Soluble sugars:

(1)

$$\text{Initial sugar quantity } [\mu\text{mol g}^{-1} \text{ of FW}] = \frac{FSC \times FV \times L}{LCF}$$

Where:

Final sugar concentration:

FSC = Sugar peak area [nC] / Slope of standard curve for this sugar [nC = f(sugar concentration)]

Final volume of resuspension:

$$FV = 2.5 \times 10^{-4} \text{ L}$$

Lost correction factor:

LCF = Standard lactose peak area for a final concentration of 100 μM / Actual lactose peak area

**A final concentration of 100 μM lactose would have been expected if adding 25 μL internal standard and not diluting samples before running. Since Dionex optimal sensor concentrations from 1 to 100 μM , and that kernel samples contains high quantity of lactose, we used a 1/100 and increase initial lactose to sugar ratio by 100.*

Lactose-Sugar Ratio:

$$L / S \text{ Ratio} = 10$$

(2) Glucose from starch digestion:

$$\text{Initial glucose quantity } [\mu\text{mol g}^{-1} \text{ of FW}] = \frac{FGC \times FV \times L}{LCF}$$

Where:

Final glucose concentration:

FGC = Glucose peak area [nC] / Slope of standard curve for glucose [nC = f(glucose concentration)]

Final volume of resuspension:

$$FV = 10^{-3} \text{ L}$$

Lactose-Glucose Ratio:

$$L / G \text{ Ratio} = 10$$

d data analysed using the

$$\frac{LCF \times L / S \text{ Ratio}}{FW}$$

concentration)]

*ose peak area **
of 1 mM of lactose as an
sitivity applies to sugar
sugars, we had to dilute them to

$$\frac{LCF \times L / G \text{ Ratio}}{FW}$$

se concentration)]

Supplemental table S1: TPS and TPP genes names and accession numbers in maize, rice, Arabidopsis and poplar

Specie	Gene name	Accecion number in PLAZA	Accecion number in other databases	Database		
Maize	ZmTPSI.1.1_tps1	ZM08G19270	GRMZM2G068943_T01	www.maizesequence.org		
	ZmTPSI.1.2	ZM06G28170	GRMZM2G001304_T01			
	ZmTPSII.2.1	ZM07G13460	GRMZM2G019183_T02			
	ZmTPSII.2.2	ZM01G08410	GRMZM2G099860_T01			
	ZmTPSII.3.1	ZM03G31900	GRMZM2G304274_T01			
	ZmTPSII.3.2	ZM03G31920	GRMZM2G123277_T01			
	ZmTPSII.3.3	ZM08G19270	GRMZM2G118462_T01			
	ZmTPSII.4.1	ZM02G30010	GRMZM2G527891_T01			
	ZmTPSII.4.2	ZM01G39130	GRMZM2G008226_T01			
	ZmTPSII.4.3	ZM04G13350	GRMZM2G366659_T01			
	ZmTPSII.5.1	ZM04G11490	GRMZM2G007736_T02			
	ZmTPSII.5.2	ZM01G37720	GRMZM2G079928_T01			
	ZmTPSII.5.3	ZM04G26220	GRMZM2G312521_T01			
	ZmTPSII.5.4	ZM05G42890	GRMZM2G122231_T01			
	ZmTPPA.1	ZM09G18330	GRMZM2G178546_T01			
	ZmTPPA.3	ZM05G35700	GRMZM2G112830_T01			
	ZmTPPB.1.1	ZM01G39020	GRMZM2G347280_T01			
	ZmTPPB.1.2	ZM02G29850	GRMZM2G140078_T01			
	ZmTPPB.1.3	ZM07G12490	GRMZM2G174396_T01			
	ZmTPPB.1.4	ZM05G40760	GRMZM2G055150_T01			
	ZmTPPB.1.5	ZM04G27640	GRMZM2G151044_T01			
	ZmTPPB.1.6	ZM09G00990	GRMZM2G080354_T01			
	ZmTPPB.2.1_ramora3	ZM07G27620	GRMZM2G014729_T01			
	ZmTPPB.2.2	ZM02G39290	GRMZM2G117564_T01			
	ZmTPPB.2.3	ZM07G27610	GRMZM5G840145_T01			
	Rice	OsTPS1	OS05G44210		HM050424	Genebank
		OsTPS2	OS01G54560		HM050425	
		OsTPS3	OS01G53000		HM050426	
		OsTPS4	OS03G12360		HM050427	
		OsTPS5	OS02G54820		HM050428	
OsTPS6		OS05G44100	HM050434			
OsTPS7		OS08G31980	HM050429			
OsTPS8		OS08G34580	HM050430			
OsTPS9		OS09G25890	HM050431			
OsTPS10		OS09G23350	HM050432			
OsTPS11		OS09G20990	HM050433			
OsTPP1		OS02G44230	AB120515			

OsTPP2	OS10G40550	AB277360
OsTPP3	OS07G43160	NM_001066861
OsTPP4	OS02G51680	NM_001054678
OsTPP5	OS04G46760	TPP5_ORYSJ
OsTPP6	OS08G31630	TPP6_ORYSJ
OsTPP7	OS09G20390	BAD25928
OsTPP8	OS06G11840	BAD37685
OsTPP9	OS03G26910	AAT78804
OsTPP10	OS07G30160	NP_001059655
OsTPP11	OS02G44235	<i>none</i>
OsTPP12	OS10G40555	Osl_34594
OsTPP13	OS12G09060	LOC_Os12g09060

Arabidops

AtTPS1	AT1G78580
AtTPS2	AT1G16980
AtTPS3	AT1G17000
AtTPS4	AT4G27550
AtTPS5	AT4G17770
AtTPS6	AT1G68020
AtTPS7	AT1G06410
AtTPS8	AT1G70290
AtTPS9	AT1G23870
AtTPS10	AT1G60140
AtTPS11	AT2G18700

AtTPPA	AT5G51460
AtTPPB	AT1G78090
AtTPPC	AT1G22210
AtTPPD	AT1G35910
AtTPPE	AT2G22190
AtTPPF	AT4G12430
AtTPPG	AT4G22590
AtTPPH	AT4G39770
AtTPPI	AT5G10100
AtTPPJ	AT5G65140

Poplar

PtTPS1	PT11G09730
PtTPS2	PT01G38460
PtTPS3	PT03G08380
PtTPS4	PT08G13400
PtTPS5	PT01G02000
PtTPS6	PT10G10370
PtTPS7	PT11G06720
PtTPS8	PT04G05810
PtTPS9	PT12G07420
PtTPS10	PT15G07580
PtTPS11	PT06G17480

PtTPS12	PT18G09730
PtTPS14	PT04G06150
PtTPPA.1.1	PT12G13940
PtTPPA.1.2	PT15G13860
PtTPPA.1.3	PT01G00080
PtTPPA.1.4	PT03G10240
PtTPPB.1.1	PT05G15960
PtTPPB.1.2	PT02G09320
PtTPPB.1.3	PT05G07700
PtTPPB.1.4	PT07G05480

Supplemental table S2: Maize TPS, TPP, SnRK1 targets and reference genes RT-qPCR primers sequence, product size and efficiency

Gene	Gene accession number	Primers sequence	Product size (bp)	Efficiency
ZmTPSI.1.1_tps1	GRMZM2G068943	ACAGAGCTACACCCGTAGCTAGTCA	107	1.81
		TCCTTTATCCTTTCCATTTGCTA		
ZmTPSII.2.1	GRMZM2G019183	AGCTACGGTCAGTCCCTCAACC	116	1.84
		GAAGATATCCATGTCATCAACACCA		
ZmTPSII.3.2	GRMZM2G123277	GCATCGGCGATGATAGGTCC	210	1.99
		AATCAGATTCCAGTTCAGCTCCAGT		
ZmTPSII.3.3	GRMZM2G118462	TTTGAAAATATTGCTGATATCATTGG	233	2
		GATTGTTTCGTCACCAATATCAAGTG		
ZmTPSII.4.1	GRMZM2G527891	CTCCAAGCGCTGAACTTATCTCTAC	216	1.92
		GCTTCCATTTCAGAATAAATACCTGAGA		
ZmTPSII.4.2	GRMZM2G008226	ATTTCTTGATTACGATGGCACACTT	185	1.97
		CCGCTAGACCAAGCTTCTCACAC		
ZmTPSII.4.3	GRMZM2G366659	TGTGAAGTGTGGCCATTATATCGTA	98	1.93
		CGTTGTTGTTGGCCAGTGCT		
ZmTPSII.5.3	GRMZM2G312521	ATGTTTGGACCACCTTTGTATATGG	100	1.71
		CGGAGTGAATGAATCAACTTCTCTT		
ZmTPSII.5.4	GRMZM2G122231	CCATGGGATACCTCCGGG	113	2
		CTCTCCTTGTGCTCGATGTAGGAG		
ZmTPPA.1	GRMZM2G178546	GGCGGAAGATGACTATAAAAAGGTT	156	2.05
		AGCGATTCAAGTAAAACTCCACAG		
ZmTPPA.3	GRMZM2G112830	GTCACCTGTATCACCAGATCC	157	1.95
		ATTGACAAGGACCTCCTCGATTTTA		
ZmTPPB.1.3	GRMZM2G174396	GCCAAGGCCTCCTCTTCTTCT	158	2.11
		CAGAACCTGTTGTTCTCCACCTTG		
ZmβGal	ZM03G38190	GGATTGCCAGGGTTACAGGA	100	2.07
		CTAACCACCTTCTCCATGCAAGTCT		
ZmAKINβ	ZM09G22070	GTTTGCTGTTACAGAGAGCCAAGG	95	2.12
		TTTCATTCTGGGATGGGATG		
ZmARG10 *	ZM10G26580	CAACCACGAGACTTTGCTTCTAAAC	100	2.03
		CTAGGCAGGAAAAGTAAAAAGGAT		
ZmMDH	ZM04G14160	ATGGTTGCTGTTTGTCTAATTG	90	2.11
		GTGTAAATAAGGCCTGGTTCAGAAA		
ZmbZIP11	ZM04G40980	GTGTACTGTGTCACCTCCACTCCAAC	92	1.93
		ATTGTATGGTGCACCTTCTTTGTTT		
ZmDPS *	ZM06G24060	CGGCTCAGGACTCCCATTT	87	2.05
		GCCGAGGCTTGAGATTGATAG		
ZmEF1α-1	GRMZM2G153541 (at1g07920)	AATCTCTGGTTTTGAAGGTGACAAC	230	1.9
		CAAAAGTAACAACCATACCAGGCTTA		
ZmEF1α-2	GRMZM2G343543 (at5g60390)_1	AAGACTTATCCGAACATCTGGTGAG	163	2.05
		AGATTTAAGCGCAAGAGAAATTTGA		
ZmEF1α-3	GRMZM2G112158 (at1g07940)	ACAGATGAAGTTGCTAAATCCAAGC	170	2.05
		GTACTIONTTCAGGAAGCAGGTCAT		
ZmPP2AA2-2	GRMZM2G122135 (at3g25800)_3	TACCTGTAATTTGTTGGGCTTTTA	151	1.97
		TACGTGTTGTGCTCCTGCTCAATTAT		
ZmCACS	GRMZM2G331032 (at5g46630)	CTGGGATTAATGACAAGATTGGAC	201	2.14
		ACCCTCTGTGATTCGATACTTCATC		
ZmCDC27	GRMZM2G392710 (at2g20000)	GATGGAAGATCCTTTGAGCAAGATA	164	2
		TTTCTGTAGACTTCCAGTGCTTCCT		