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The Role of Trehalose 6-Phosphate in Crop Yield and Resilience[1][OPEN]

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Significant increases in global food security require improving crop yields in favorable and poor conditions alike. However, it is challenging to increase both crop yield potential and yield resilience simultaneously, since the mechanisms that determine productivity and stress tolerance are typically inversely related. Carbon allocation and use may be amenable to improving yields in a range of conditions. The interaction between trehalose 6-phosphate (T6P) and SnRK1 (SNF1-related/AMPK protein kinases) significantly affects the regulation of carbon allocation and utilization in plants. Targeting T6P appropriately to certain cell types, tissue types, and developmental stages results in an increase in both yield potential and resilience. Increasing T6P levels promotes flux through biosynthetic pathways associated with growth and yield, whereas decreasing T6P levels promotes the mobilization of carbon reserves and the movement of carbon associated with stress responses. Genetic modification, gene discovery through quantitative trait locus mapping, and chemical intervention approaches have been used to modify the T6P pathway and improve crop performance under favorable conditions, drought, and flooding in the three main food security crops: wheat (*Triticum aestivum*), maize (*Zea mays*), and rice (*Oryza sativa*). Interestingly, both *trehalose phosphate synthase* (*TPS*) and *trehalose phosphate phosphatase* (*TPP*) genes are associated with maize domestication. A phylogenetic comparison of wheat *TPS* and *TPP* with eudicots and other cereals shows strong distinctions in wheat in both gene families. This Update highlights recent research examining the potential of the trehalose pathway in crop improvement and highlights an emerging strategy to increase cereal yields by targeting T6P in reproductive tissue.

SUC AND TREHALOSE: THE YIN AND YANG OF CROP IMPROVEMENT

Plants are the only organisms that synthesize both nonreducing disaccharides, trehalose and Suc. The ubiquity of both pathways in plants has been known for less than 20 years and was a major revelation for those working on carbon metabolism as well as plant scientists in general, given the range of processes affected by the trehalose pathway. Plant metabolism is highly regulated. Part of this regulation is through T6P signaling that regulates metabolism in the light of carbon availability and reprograms metabolism between

ADVANCES

- T6P can be targeted to improve yield potential and resilience to diverse stresses by regulating whole-plant carbohydrate allocation and utilization.
- Promoting carbohydrate allocation and utilization in sinks by optimizing T6P increases photosynthesis.
- Increasing T6P promotes biosynthetic pathways associated with grain yield, such as starch synthesis, while decreasing T6P promotes resource mobilization and changes in sucrose allocation, enabling better performance under abiotic stress.
- Expressing a *TPP* gene with a *MAD6S* promoter in maize shows how drought tolerance can be improved in the field without imposing a yield penalty under well-watered conditions.
- Genetic variation in *TPP* genes is associated with traits including grain weight and germination under anaerobic conditions.
- Chemical intervention of T6P increases grain size when applied to grain and promotes recovery of vegetative tissue after drought, most likely through gene priming for these traits.

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anabolic or catabolic pathways depending on the carbohydrate status of the plant. This discovery also is significant for understanding the regulation of growth and development by carbon supply. Furthermore, the trehalose pathway may widely impact crop improvement. Crops are not yet optimized to maximize their biosynthetic pathways for yield in sinks and growth recovery that are promoted by high T6P or for the mobilization of reserves and sugar transport that can enable resilience that are promoted by low T6P.

Both the trehalose and Suc biosynthesis pathways draw from a pool of core metabolites, from which the carbon skeletons for all cellular components also are made (Paul et al., 2008). Flux through the trehalose pathway is more than a 1,000-fold lower than that through the Suc pathway. The low abundance of both T6P and trehalose meant that T6P and trehalose did not show up in analytical methods that were used to measure other sugars and sugar phosphates. It was not until more sensitive methods were developed, as in potato (*Solanum tuberosum*; Roessner et al., 2000), that T6P and trehalose could be detected more routinely. Subsequently, several laboratories have established procedures to measure the abundance of T6P and trehalose (Lunn et al., 2006; Delatte et al., 2009, 2011; Carillo et al., 2013; Mata et al., 2016). The capacity to synthesize trehalose in plants began to become apparent as the associated plant genes were identified (Blázquez et al., 1998; Vogel et al., 1998). Subsequent publication of the [AU : 1] Arabidopsis (*Arabidopsis thaliana*) genome showed an abundance of both *TPS* and *TPP* gene families, with 11 and 10 members, respectively (Leyman et al., 2001).

It is likely that T6P is a specific signal indicating Suc abundance (Lunn et al., 2006; Nunes et al., 2013a). T6P and Suc levels are correlated in many tissues, as in Arabidopsis and wheat (Lunn et al., 2006; Martinez-Barajas et al., 2011), with the abundance of T6P being 3 orders of magnitude lower than that of Suc. Increases in Suc from an endogenous or exogenous source lead to a rapid induction of T6P in Arabidopsis (Lunn et al., [AU : 2] 2006; Nunes et al., 2013). The mechanistic details of this induction are not completely known but could involve Suc regulation of *TPS1* expression, which increases linearly in response to Suc at low endogenous Suc levels (Nunes et al., 2013c). *TPS5* is strongly induced by Suc while other *TPSs* (*TPS8*–*TPS11*) are strongly repressed by Suc, yet their roles in regulating T6P levels remain unknown. Heterologous expression of *AtTPS2* and *AtTPS4* in the *tps1* and *tps2* yeast mutants restores the yeast's ability to synthesize T6P and trehalose (Delorge et al., 2015). However, these *TPSs* are not widespread in species outside of the Brassicaceae, so the broader significance of *TPS2* and *TPS4* in T6P synthesis is unclear. The transcription factor *bZIP11* induced by Suc starvation induces the promoters of *TPP5* and *TPP6* and of trehalase (*TRE1*; Ma et al., 2011), which may be important in controlling T6P levels under low carbon supply. All *TPPs* are likely catalytic (Vandesteene et al., 2012), whereas only *TPS1* and *AtTPS2* and *AtTPS4* out of 11 TPSs in Arabidopsis are catalytic. The functions of the other TPSs have yet to be resolved.

Interestingly, prior to the discovery of T6P, research on Glc signaling through hexokinase already had shown how a sugar signaling mechanism might operate. Hexokinase senses Glc availability via a mechanism that is distinct from its catalytic function (Jang et al., 1997). Plants do not synthesize Glc directly in photosynthesis; Glc is a product of the catabolism of Suc, starch, and cell wall carbohydrates, whereas Suc is made directly through photosynthesis and, as transportable carbon, is the starting point for the provision of carbon for the synthesis of all plant components. Hence, Glc and Suc signaling are likely to represent different avenues for crop improvement. A mechanism that senses Suc and regulates the use of Suc can potentially integrate photosynthesis with the use of Suc to coordinate source and sink activities, a powerful means of crop improvement already exemplified (Paul et al., 2017; see below).

CONSTITUTIVE EXPRESSION OF THE TREHALOSE PATHWAY IN TRANSGENIC PLANTS

Shortly before the discovery of active plant trehalose pathway enzymes (Blázquez et al., 1998; Vogel et al., 1998), *Escherichia coli* trehalose pathway genes had been transformed into plants to use plants as a vehicle for trehalose production and improve drought tolerance through the accumulation of trehalose (Goddijn et al., 1997). Trehalose was thought to be a key component that protects resurrection plants, such as *Selaginella* spp. (Zentella et al., 1999; Iturriaga et al., 2000) and *Myrothamnus flabellifolius* (Müller et al., 1995), from desiccation as an osmolyte. As a nonreducing disaccharide, trehalose is stable and unreactive and accumulates to high levels in fungi, bacteria, insects, and arthropods, where it doubles as a carbon store and protection compound in some cases and was proposed as a target to improve drought tolerance in crops. The *E. coli* genes *otsA* encoding TPS and *otsB* encoding TPP were heterologously expressed in the model plants tobacco (*Nicotiana tabacum*; Goddijn et al., 1997) and Arabidopsis (Schluepmann et al., 2003). Very little trehalose accumulated, but the transgenic plants displayed healthy and robust phenotypes (Schluepmann et al., 2003; Pellny et al., 2004). This contrasted with the phenotypes of many other transgenic plants where metabolism had been targeted, which either had impaired growth and development or had no effect on phenotype compared with wild-type plants (Paul et al., 2001).

To explain this, it was proposed that the trehalose pathway was engaging somehow with endogenous regulatory or signaling mechanisms that linked carbon supply with the regulation of plant growth and development. This also represented a strategy to modify carbon metabolism and resource allocation to improve crop yield (Paul et al., 2001).

Constitutive heterologous expression of trehalose pathway genes in plants was a valuable tool to establish the importance of the pathway, particularly of T6P, as a powerful regulator linking metabolism with growth and development. For example, the earliest demonstration of the requirement of T6P for carbohydrate utilization in plants (Schluepmann et al., 2003) links to abscisic acid and auxin signaling (Avonce et al., 2004; Paul et al., 2010) as well as the activation of starch synthesis (Kolbe et al., 2005) and breakdown (low T6P; Martins et al., 2013). The wide-ranging impact on metabolic pathways through inhibition of the protein kinase SnRK1 (Zhang et al., 2009) also was shown, which is discussed below. Experiments using an ethanol-inducible promoter also have shown that organic and amino acid metabolism are regulated through the posttranslational activation of nitrate reductase and phospho*enol*pyruvate carboxylase in Arabidopsis rosettes (Figueroa et al., 2016). There also have been several reports showing improvements in stress tolerance in plants with constitutively expressed trehalose pathway genes, with a notable example in rice (Garg et al., 2002), but this has not transferred to field cultivation. It has not been possible to increase trehalose contents to provide significant osmoprotection, but trehalose may promote the autophagy that is associated with drought tolerance (Williams et al., 2015). Alternatively, changes in T6P levels may be responsible for improved stress tolerance, although measurements of T6P rarely accompany studies, which limits our understanding of the mechanistic basis of enhanced drought tolerance obtained by modifying the pathway. Interestingly, a study of trehalose contents in the drought-tolerant crops cassava (*Manihot esculenta*), *Jatropha curcas*, and castor bean (*Ricinus communis*) showed far higher levels of trehalose, particularly in leaves, than has been reported generally. This study reported up to 3 μmol trehalose g^{-1} fresh weight (Han et al., 2016) compared with values that are usually more than 100-fold lower, in the tens of nmol g^{-1} fresh weight. Trehalose levels also appeared to be related to dehydration stress tolerance in detached leaves, and trehalose increased in response to osmotic stress. It is possible that a wider survey of species with different environmental adaptions could show a stronger link between trehalose levels and drought tolerance than has been appreciated more generally. Levels of trehalose itself could reflect flux through the pathway rather than necessarily implicating a direct involvement of trehalose in drought tolerance, which may be regulated by T6P. Interestingly, a comparison of two *Selaginella* spp. that vary in drought tolerance showed that the drought-resistant species contained less trehalose than the susceptible species (Pampurova et al., 2014), so the relationship between trehalose accumulation and drought tolerance is not at all clear. In yeast, the TPS1 protein itself, and not trehalose, is proposed to mediate stress tolerance (Petitjean et al., 2016).

For a major regulatory pathway, while constitutive expression of transgenes has given valuable new insight, constitutive promoters are too much of a blunt instrument with which to understand the subtleties of the cell, including the tissue- and development-specific nature of the regulation of metabolism by T6P. This is crucial not only for a mechanistic understanding but also for crop improvement. *TPS* and *TPP* gene expression is under strong cell-, tissue-, development-, and environment-specific regulation (Paul et al., 2008; Schluepmann and Paul, 2009). Martinez-Barajas et al. (2011) showed strong tissue- and development-specific changes in T6P contents in wheat grain during development. T6P is found in both maternal and paternal tissues at high levels up to 10 d after anthesis, but it is restricted to the endosperm beyond this period despite the sustained levels of Suc in maternal tissues. Hence, the role of T6P as a signal of Suc availability (Lunn et al., 2006) has a strong developmental context. It is highly unlikely that superior crops with a genetically modified trehalose pathway will emerge without appreciating and targeting T6P-dependent regulation in a cell- and development-specific manner.

TARGETED EXPRESSION OF THE TREHALOSE PATHWAY IN TRANSGENIC MAIZE

Targeted expression (as opposed to the constitutive expression discussed above) of a *TPP* gene in maize is a striking example of how the pathway might be modified to improve crop performance. Water availability is the primary factor affecting crop yields worldwide. For many crops, including cereals, water deficit during the transition to reproductive development has the greatest impact on yield by reducing grain set and, hence, final grain numbers. For example, maize kernel numbers can be largely preserved during drought if they are supplied with Suc (Zinselmeier et al., 1995; Boyer and Westgate, 2004). Combined with emerging knowledge about the function of T6P in regulating carbohydrate use in plants (Schluepmann et al., 2003) and the known relationship between the trehalose pathway and drought tolerance (Garg et al., 2002), Nuccio et al. (2015) put forward a strategy to target the pathway in reproductive tissue during the flowering period. When using a *MADS6* promoter, which is active during flowering in reproductive tissue, to drive the expression of a *TPP* gene, substantial improvements in grain set and yields were achieved during droughts of different severities during flowering (Nuccio et al., 2015). Furthermore, yield was increased even without drought. It is likely that grain abortion is a survival strategy to ensure that at least some kernels survive when resources are limiting. The plant has no way of knowing how severe the drought will be, and in many cases, more grains are aborted than is necessary. Increasing the Suc contents of developing kernels with this genetic modification approach represents a strategy to achieve higher yields under drought and is one of the few examples where genetic modification has improved drought tolerance that has been thoroughly tested in field conditions with

no yield penalty under optimal hydration. Interestingly, drought tolerance in this example is improved by targeting carbon allocation rather than directly targeting water use efficiency. This work to target T6P in maize reproductive tissue began before more recent insights on the mechanistic mode of action of T6P through SnRK1 signaling were available (Zhang et al., 2009).

MECHANISTIC BASIS OF CROP YIELD IMPROVEMENT FROM TARGETING T6P

Much of the fundamental work on T6P signaling has been conducted in Arabidopsis. This has provided a good framework for understanding the function of the pathway and will continue to be an invaluable tool. In fact, an ideal discovery/crop improvement program would include Arabidopsis alongside crops, given the Arabidopsis genetic resources and the ease of cultivation and experimentation in this species. Many of the discoveries regarding the function of T6P in Arabidopsis also will hold true in crops because of the central and conserved function of the pathway. However, a major limitation of Arabidopsis is that it does not have any large sinks. Sink tissues of tubers, fruit, seeds, and grain form the majority of harvested crop produce. Furthermore, because of the absence of large sinks in Arabidopsis, source-sink relations may be less sophisticated in Arabidopsis than in crops where a large proportion of carbon is partitioned to harvested sinks. Hence, this process needs to be studied in crops for crop improvement and to appreciate the full role of T6P in plant processes. For example, while some of the general principles behind targeting T6P to improve drought tolerance in maize (Nuccio et al., 2015) came from understanding that T6P regulates carbon utilization in Arabidopsis (Schluepmann et al., 2003), this had to accompany knowledge of the development of reproductive sinks in maize.

One notable difference between Arabidopsis and cereals is the level of T6P. In Arabidopsis, T6P levels can reach 10 nmol g^{-1} fresh weight in seedlings (Nunes et al., 2013a), although typically levels are 10-fold lower and are another order of magnitude lower in rosettes (Lunn et al., 2006). Additionally, 119 nmol T6P g^{-1} fresh weight has been reported in the wheat grain endosperm (Martinez-Barajas et al., 2011). Maize kernels contain up to 50 nmol T6P g^{-1} fresh weight (Nuccio et al., 2015; Bledsoe et al., 2017). There are currently no T6P measurements in rice grain, although the concentration of T6P is expected to be as high as in other cereals. A T6P:Suc nexus has been proposed, which may differ across tissues and species (Yadav et al., 2014). The basis of this nexus is that Suc induces T6P biosynthesis, which then drives down Suc levels by promoting Suc consumption through metabolism, storage, growth, and development. In crops, domestication may indeed have changed this nexus. Interestingly, while T6P appears to consistently correlate with Suc in Arabidopsis, it does not always do so in wheat when T6P levels are resolved in specific tissue levels during various developmental stages. In wheat grain, T6P is found at high levels in paternal and maternal tissues during early development up to 10 d after anthesis. After this stage, T6P becomes confined to the endosperm, even though Suc levels are still high, with pericarp tissues showing that development and tissue type can override direct effects of Suc on T6P levels (Martinez-Barajas et al., 2011). In maize, after returning culture kernels from starvation to Suc-rich conditions, direct correlations between Suc and T6P levels become much weaker, suggesting a complex regulation of T6P levels beyond the effects of Suc that are not yet fully understood (Bledsoe et al., 2017). It is possible that there is some deregulation of the T6P:Suc nexus in crops that have been selected for inbreeding, which may be necessary for high yields. In wheat and maize, the T6P-Suc ratio is lower than in Arabidopsis. It is possible that the catalytic machinery for the synthesis and breakdown of T6P, sensing T6P, or the downstream interaction with SnRK1 gives rise to a lower T6P:Suc nexus.

Inhibition of SnRK1 by T6P provides a framework for understanding the mode of action of T6P and for many of the wide-ranging effects of modifying T6P in plants (Zhang et al., 2009), including providing a basis for the T6P:Suc nexus. SnRK1 is a member of the conserved AMPK, SNF1-related protein kinases that regulate energy- and carbon availability-related processes in all eukaryotic organisms. SnRK1 regulates enzyme activities directly by changing their phosphorylation states (Halford et al., 2003) and regulates gene expression (Baena-González et al., 2007). The bZIP11 transcription factor may be particularly important in this regard (Delatte et al., 2011; Ma et al., 2011). T6P inhibits the catalytic activity of SnRK1 to relieve the inhibition of growth and development by SnRK1, thus enabling growth to proceed in the presence of adequate carbon. Biosynthetic pathway and growth genes that are repressed by SnRK1 are derepressed by T6P, whereas stress and catabolic pathway genes that are induced by SnRK1 are repressed by T6P. In this way, T6P provides the licensing factor, as it has been described, for Suc utilization in plants (Smeekens, 2015). The inhibition of SnRK1 by T6P has been shown in Arabidopsis in vitro protein kinase assays that measure SnRK1 catalytic activity with and without T6P (Zhang et al., 2009) and can be inferred in SnRK1 overexpression lines, which rescue plant growth on high trehalose and, hence, overcome the T6P inhibition of SnRK1 in these conditions (Delatte et al., 2011). Catalytic assays also show SnRK1 inhibition by T6P in crops, such as sugarcane (*Saccharum officinarum*; Wu and Birch, 2010), potato (Debast et al., 2011), wheat (Martinez-Barajas et al., 2011), maize (Nuccio et al., 2015), and cucumber (*Cucumis sativus*; Zhang et al., 2015). SnRK1 marker genes were identified in the pioneering work of Baena-González et al. (2007), and these were shown to change in a manner consistent with T6P-mediated changes in SnRK1 activity in vivo in transgenic Arabidopsis with

altered T6P content (Zhang et al., 2009). A similar pattern emerged when Arabidopsis seedlings were exposed to different environmental treatments that affected T6P levels (Nunes et al., 2013). Relationships between SnRK1 marker genes and T6P also have been shown in wheat (Martinez-Barajas et al., 2011) and maize (Henry et al., 2015; Bledsoe et al., 2017). T6P inhibits SnRK1 in all actively growing tissues, while in mature leaves, SnRK1 is largely insensitive to inhibition by T6P (Zhang et al., 2009). This fits a paradigm where T6P and SnRK1 coordinate Suc usage with anabolic growth and development. In Arabidopsis seedlings, the inhibition constant (K_i) of T6P for SnRK1 is around 5 μM (Nunes et al., 2013b), whereas in wheat and maize grain, it is about 50 μM (Martinez-Barajas et al., 2011; Nuccio et al., 2015). For noncompetitive inhibitors, such as T6P, K_i is the concentration of inhibitor required for 50% inhibition. The different SnRK1 K_i levels in crops compared with Arabidopsis could be due to crop domestication.

Recently, important insight was obtained on how changes in T6P levels in transgenic maize (Nuccio et al., 2015) result in a larger grain set and higher yields (Oszvald et al., 2018). The dissection of female reproductive tissues expressing the *MADS6-TPP* construct showed that changes in T6P produce outcomes that are highly tissue specific. Two- to 3-fold decreases in T6P produced by this construct resulted in similar changes in gene expression for metabolic pathways in component tissues of developing maize cobs, down-regulating primary metabolic pathways (Suc, starch, and amino acids) but up-regulating secondary metabolic pathways (lipids and endogenous trehalose). However, this produced different effects on metabolite distribution in florets compared with the pith tissue that supplies the florets. Florets accumulated Suc and amino acids at the expense of the pith. This was related to the up-regulation of several *SWEET* genes in the pith and florets and to the maintenance of higher rates of photosynthesis for longer in leaves. This could form the basis for a molecular mechanism of source-sink relations. The transgene was found to localize to the phloem vasculature and companion cells. Altering T6P in the phloem could mediate changes in Suc transport via T6P/SnRK1-regulated SWEET proteins. This also stimulated photosynthesis in leaves (Oszvald et al., 2018). This provides an exciting model for source-sink relations that can be tested and developed in crop improvement (Fig. 1). In support of this, TPS1 and bZIP11 expression is associated with the phloem vasculature [AU : 3] (Genevestigator [https://genevestigator.com/gv/]; Lastdrager et al., 2014). Oszvald et al. (2018) also showed changes in SnRK1 activity, SnRK1 subunit composition, and the expression of SnRK1 marker genes, together with striking changes in endogenous trehalose pathway genes, which suggest mutual T6P/SnRK1 regulation as part of a feedback loop.

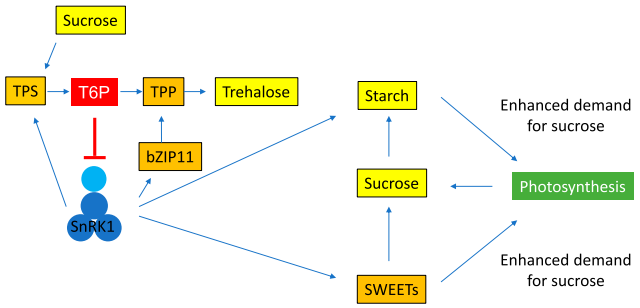

Figure 1. Mechanistic framework for the regulation of source-sink relations mediated by T6P/SnRK1. T6P tracks Suc levels. T6P inhibits SnRK1, which regulates key genes involved in the allocation (SWEETs) and use (starch biosynthesis) of Suc. The enhanced use of Suc stimulates carbon fixed in photosynthesis. A feedback loop likely operates where SnRK1 phosphorylates bZIP11, which regulates the expression of TPPs; other TPSs are regulated by SnRK1 as established SnRK1 marker genes. Proteins are shown in orange boxes, and carbohydrates are shown in yellow boxes.

NATURAL VARIATION IN TPP GENES UNDERLYING IMPORTANT CROP TRAITS

Ramosa3 (*RA3*) was one of the earliest examples of a trehalose pathway gene affecting a crop trait (Satoh-Nagasawa et al., 2006). Maize has both male and female reproductive structures with different architectures that were selected during maize domestication to enhance its utility as an agricultural crop. The male inflorescence, or tassel, has long branches at its base and a central spike that bears shorter branches containing spikelet pairs. The female inflorescences are positioned laterally and contain only short branches, a trait that is thought to aid in the efficient packing and harvesting of seeds. *RA3* is required for this specialized architecture, because *ra3* mutant tassels have additional long branches and *ra3* mutant ears have abnormally long branches at their bases. *RA3* encodes a TPP that regulates inflorescence branching and is expressed in discrete domains subtending axillary inflorescence meristems. *RA3* may regulate inflorescence branching by the modification of T6P or a target of T6P that moves into axillary meristems. However, alteration of T6P concentrations by *RA3* has not been shown because of the difficulties in measuring T6P in very small discrete tissue samples. Alternatively, *RA3* itself may have a transcriptional regulatory function.

A TPP represents a quantitative trait locus in rice for germination under anaerobic conditions (Kretzschmar et al., 2015). The primary way of cultivating rice is in flooded paddy fields, and the ability to germinate well in such conditions has been a sought-after trait. Directly seeding rice into flooded fields would remove the necessity for labor-intensive transplantation of rice plants. The efficient use of starch reserves is crucial in coping with energy starvation and maintaining growth under anaerobic germination. Kretzschmar et al. (2015) identified a functional *TPP7* gene in a genomic region, qAG-

9-2, that is associated with the tolerance of anaerobis during germination. *TPP7* likely contributes to anaerobic germination tolerance by modulating and decreasing local T6P levels, which leads to enhanced starch mobilization through amylase activation. The flux of sugars toward the sink is promoted, providing the energy and carbon necessary for coleoptile growth under anaerobic conditions.

A wheat TPP was found recently to be associated with grain weight in bread wheat (Zhang et al., 2017). Using recombinant inbred lines derived from parents with high and low thousand grain weight (TGW), a [AU : 4] single-nucleotide polymorphism in a promoter region of a *TPP* gene (*TaTPP-6AL1*) was identified that is significantly associated with TGW. Differential expression and alteration of T6P levels, likely in the reproductive tissues, may confer TGW variation in wheat. This gene could be used as a selection marker to increase wheat yield potential.

So far, *TPP* genes are the only trehalose pathway genes that are known to affect crop improvement. No *TPS* genes have been associated with crop traits. Interestingly, based on the genome-wide resequencing of 75 wild landrace and improved maize lines, both *TPS* and *TPP* genes are listed as genes associated with domestication improvement (Hufford et al., 2012). It is of great interest to determine whether closely associated genes are present in other important crops such as wheat, how domestication has affected these, and what further selection may be possible. This suggests that both TPSs and TPPs could be targeted in crop improvement programs, including those for wheat.

PHYLOGENETIC ANALYSIS OF WHEAT *TPS* AND *TPP* GENES

We conducted a phylogenetic comparison of the *TPS* and *TPP* gene families using the genome sequences

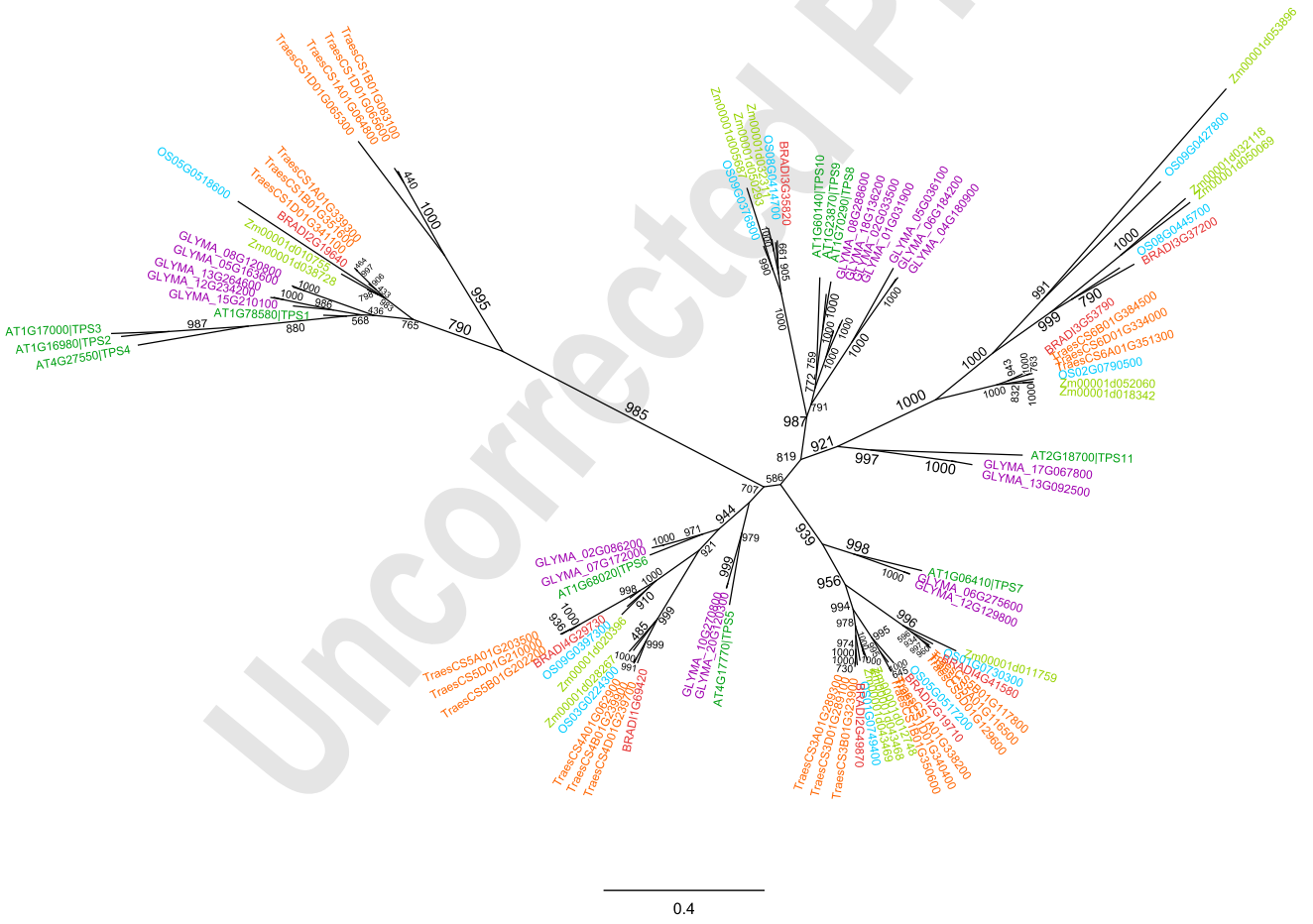

Figure 2. Phylogenetic tree of *TPS* proteins. Species are indicated as follows: Arabidopsis (AT, dark green), soybean (GLYMA, purple), *B. distachyon* (BRADI, brown), *japonica* rice (OS, light blue), maize (Zm, light green), and bread wheat (TraesCS, orange). Full-length *TPS* protein sequences were aligned using MUSCLE version 3.8.425 (Edgar, 2004) with default parameters as part of the Geneious tool suite (version 10.0.9 [https://www.geneious.com]; Kearse et al., 2012), and gaps were removed manually. Maximum likelihood trees were constructed using PhyML (Guindon et al., 2010) with 1,000 bootstraps and implementing the default LG amino acid substitution model (Le and Gascuel, 2008). The scale bar represents 0.4 amino acid substitutions per site. The tree was depicted using the tree viewer in Geneious and edited using Inkscape.

available in Ensembl Plants release 38 (http://plants.ensembl.org/index.html) for Arabidopsis, *japonica* rice, *Brachypodium distachyon*, maize, soybean (*Glycine max*), and bread wheat (Figs. 2 and 3). Arabidopsis *TPS* and *TPP* genes (Leyman et al., 2001) were used as a query to identify orthologues in these species using the Ensembl Plants BioMart portal (http://plants.ensembl.org/biomart/martview; Smedley et al., 2015). Additionally, Ensembl Plants release 38 was searched for genes annotated with the *TPP* (IPR003337) constituent domain to avoid missing more divergent genes. Genes were classified as *TPSs* if they contained a glycosyl

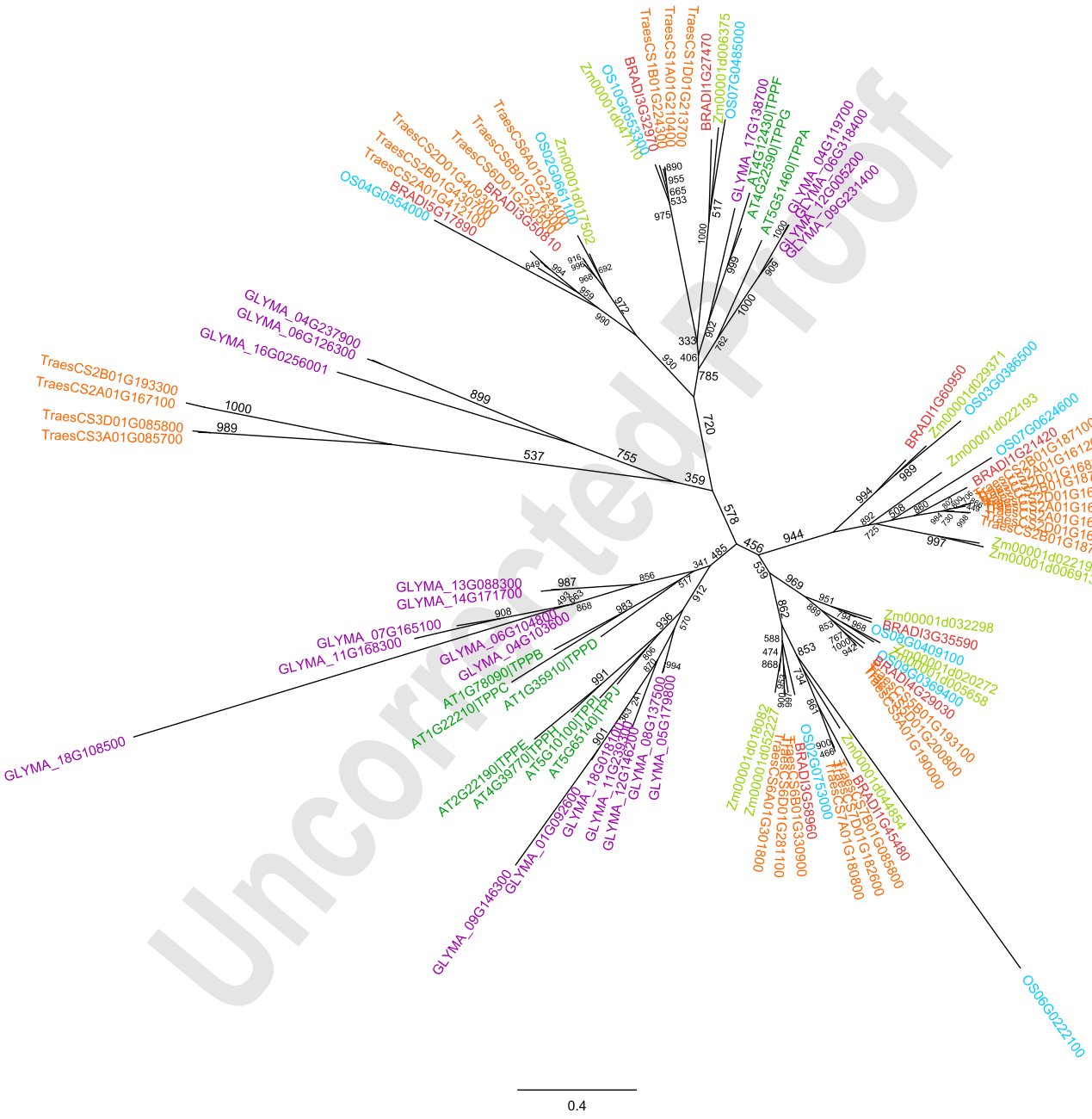

Figure 3. Phylogenetic tree of *TPP* proteins. Species are indicated as follows: Arabidopsis (AT, dark green), soybean (GLYMA, purple), *B. distachyon* (BRADI, brown), *japonica* rice (OS, light blue), maize (Zm, light green), and bread wheat (TraesCS, orange). Full-length *TPP* protein sequences were aligned using MUSCLE version 3.8.425 (Edgar, 2004) with default parameters as part of the Geneious tool suite (version 10.0.9 [https://www.geneious.com]; Kearse et al., 2012), and gaps were removed manually. Maximum likelihood trees were constructed using PhyML (Guindon et al., 2010) with 1,000 bootstraps and implementing the default LG amino acid substitution model (Le and Gascuel, 2008). The scale bar represents 0.4 amino acid substitutions per site. The tree was depicted using the tree viewer in Geneious and edited using Inkscape.

transferase family 20 (IPR001830) domain and as *TPPs* if they only had the phosphatase domain as described by Leyman et al. (2001) and Lunn (2007; Supplemental Figs. S1 and S2; Supplemental Tables S1 and S2). Using this approach, we identified 99 plant *TPS* genes and 100 *TPPs*, but manual investigation of gene length, structure, and domain composition suggested that several plant trehalose pathway genes were not complete or were incorrectly annotated in their respective genome versions (Supplemental Tables S3 and S4). Therefore, genes that were more divergent than the *E. coli otsA* and *otsB* genes that were used as outgroups (data not shown) were not included in the final analysis. Similarly, the *E. coli* genes were removed from the final tree to improve legibility. In addition, the wheat protein sequences belonging to the TGACv1 wheat reference (Clavijo et al., 2017) were improved (Supplemental Tables S5 and S6) by performing BLAST searches in an in-house TimeLogic DeCypher server (tera-blastp, max_scores = 10, e-value ≤ 1–5) against the high-confidence IWGSC RefSeq v1.0 proteins (available under Toronto agreement). Using this methodology, we have identified 25 wheat *TPS* proteins (Supplemental Tables S7 and S9) and 33 *TPPs* (Supplemental Tables S8 and S10).

The reconstructed evolutionary story is markedly different for *TPS* and *TPP* genes, but there are some common features. With a few exceptions, genes of the two eudicot species (Arabidopsis and soybean) clustered together more closely than the genes of the four monocot species, supporting the ancestral origin of the genes as divergent evolution after the monocot-eudicot separation. Within the monocots, the clusters tended to have one *B. distachyon* gene very closely related to three wheat homeologues, as expected because the former is considered the closest fully sequenced wild relative of wheat. In addition, one rice gene and two maize genes (old tetraploid) usually clustered with them. This pattern is quite consistent within the *TPS* phylogenetics tree (Fig. 2) but is more variable among the *TPPs* (Fig. 3). The *TPS* tree is divided into two main clades, as noted by several authors (Lunn, 2007; Henry et al., 2014; Xie et al., 2015; Han et al., 2016). Class I includes *TPSs* with a high number of exons (minimum of nine but usually more than 15; Supplemental Table S4A). In contrast to previous studies, we found that the Arabidopsis class I *TPS* genes did not separate into two subgroups (*AtTPS1* and *AtTPS2–AtTPS4*) but rather clustered together with five soybean genes, suggesting that expansion of the class I genes may be a feature of the eudicots, as it also happens in cassava (Han et al., 2016). Interestingly, wheat had two class I gene sets totaling seven genes that were all on chromosome 1. It seems that the most divergent set of genes had four homeologues instead of three due to a potential tandem duplication in chromosome 1D (Supplemental Table S9).

The class II *TPSs* can be divided further into four clades with distinct monocot-eudicot features. *AtTPS11* had a single orthologue in wheat, which is surprising given that *B. distachyon* had two genes in the same clade and rice and maize have even more. *AtTPS5* and *AtTPS6* formed a common clade with two wheat, two *B. distachyon*, and two maize genes. The *TPS7* gene was extended as a family in monocots, with three wheat, *B. distachyon*, and rice genes. The two maize genes (*Zm00001d043468* and *Zm00001d043469*) closest to *AtTPS7* are genes associated with domestication improvement (Table I; Fig. 2; Hufford et al., 2012). The

Table I. TPS *and* TPP *genes associated with domestication improvements in maize (from Hufford et al., 2012) and with improvements in specific crop traits in wheat, rice, and maize*

[AU : 8]

Cereal Gene	Closest Arabidopsis Gene	Putative Physiological Role	Close Gene in Wheat	Close Gene in Rice	Close Gene in Maize
Zm00001d043468 *Zm00001d043469*	*TPS7*	Improvement candidates in maize (Hufford et al., 2012); no known function	√	√	
Zm00001d032311	*TPS8* to *TPS10*	Improvement candidate in maize (Hufford et al., 2012); no known function	No	√	
TraesCS6A01G248400 *TraesCS6B01G276300* *TraesCS6D01G230500*	*TPPA, TPPF,* and *TPPG*	Likely catalytic function, associated with grain weight (Zhang et al., 2017)		√	√
Os09T0369400 TPP7	Distinct cereal TPP clade	Likely catalytic function, enables germination under anoxia through starch mobilization (Kretzschmar et al., 2015)	√		√
Zm00001d022193	Distinct cereal TPP clade	*RA3*, role in inflorescence architecture (Satoh-Nagasawa et al., 2006)	√	√	
Zm00001d032298	Distinct cereal TPP clade	Improvement candidate in maize (Hufford et al., 2012); likely catalytic function	√	√	

clade containing *TPS8* to *TPS10* appears to have had a varied fate in the different monocots, with no wheat genes, a single *B. distachyon* gene, and multiple rice and maize genes. *Zm00001d032311* is another maize gene associated with domestication improvement (Hufford et al., 2012). *TPP* genes have diversified even more, with most clades being characteristic of either monocots or eudicots (Fig. 3). Some authors (Henry et al., 2014; Xie et al., 2015; Han et al., 2016) divide *TPPs* into two clades (A and B), mainly based on the Arabidopsis *TPPs* clustering into two groups, but only clade A, which includes *AtTPPA*, *AtTPPF*, and *AtTPPG*, seems to be conserved in all species. Clade B (*AtTPPB*, *AtTPPC*, *AtTPPD*, *AtTPPE*, *AtTPPH*, *AtTPPI*, and *AtTPPJ*) was more diverse and was divided into an exclusively eudicot clade and three monocot-exclusive clades. Interestingly, one of the monocot-only clades had three wheat genes with three homeologues each, all of them on chromosome 2. *Zm00001d032298* is another gene associated with maize domestication improvement (Hufford et al., 2012). There also was an isolated clade that contained four wheat and three soybean proteins that were more related to the ancestral bacterial *otsB* protein (not shown in the tree). However, caution is required when interpreting this clade, since three of the four wheat genes were longer than average and also encoded a *Gnk2* homologue fungal resistance domain (Supplemental Tables S8 and S10).

The differing gene numbers across the species could be an evolutionary sign of the flexibility of trehalose biosynthesis genes, with each of them having specialized roles in different species, although artifacts due to problems with genome assemblies and annotations still cannot be ruled out due to the nascent nature of sequence information for wheat. A more detailed expression analysis is needed to confirm the inferred functions of these wheat genes.

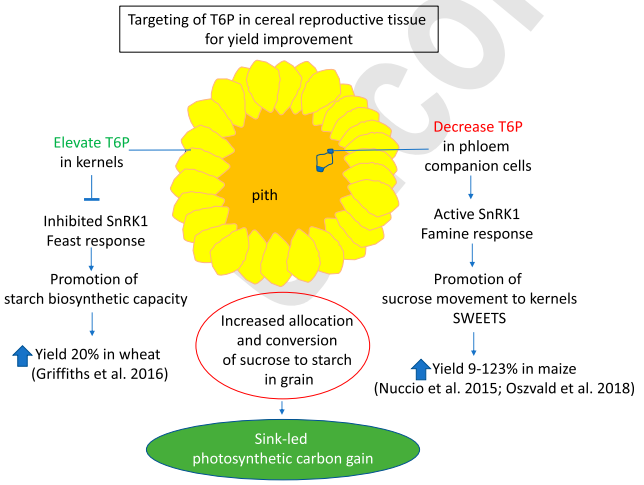

Figure 4. Model for the modification of T6P levels to increase yield in cereal reproductive tissue. Elevation of T6P levels in cereal grain increases starch biosynthesis and grain size. A decrease in T6P in phloem companion cells increases *SWEET* gene expression and Suc efflux into the grain.

CHEMICAL INTERVENTION OF T6P

Genetic methods using natural variation or genetic modification of the trehalose pathway show great promise for crop improvement. A recent complementary development is chemical intervention with T6P, which can serve as an additional tool for perturbing T6P levels, identifying new genes, and probing for further details of the mechanistic basis of T6P signaling (Griffiths et al., 2016). T6P in its native form does not cross membranes. Griffiths et al. (2016) showed that applying T6P precursors synthesized using phosphorus chemistries to attach light-labile chemical groups enabled the uptake of T6P, but these are then cleaved away in sunlight or UV light to release T6P and increase endogenous T6P levels more than 100-fold. One 1 mM [AU : 5] spray, which delivered a pulse of T6P 10 d after anthesis, had the effect of increasing grain size and yield at harvest 1 month later. Much can be gained by increasing T6P well above normal physiological limits. The role of T6P in starch synthesis and biosynthetic pathways already had been shown (Kolbe et al., 2005; Zhang et al., 2009), and the stimulation of starch synthesis by T6P suggests that the capacity for starch synthesis in grain currently imposes a limitation on wheat yields. T6P primes gene expression for starch biosynthesis, and stimulating sink capacity in such a way is a promising route to yield improvement.

T6P also stimulates biosynthesis in other ways. Transfer of plants from cold to warm results in a growth spurt following the transfer. It was shown that T6P primes gene expression for this growth stimulation (Nunes et al., 2013a). Following this precedent, the T6P precursors also were used to see if recovery from drought could be enhanced. In this instance, new

OUTSTANDING QUESTIONS

- How has domestication already changed the trehalose and SnRK1 pathways and what additional changes to T6P/ SnRK1 signaling can be made to further improve crop traits?
- What are the functions of the many *TPS* and *TPP* genes in wheat and other crops?
- What are the SnRK1, TPS, and TPP signaling complexes composed of?
- What are the T6P targets beyond SnRK1?
- What is the function of trehalose?

growth and resurrection of existing growth were stimulated by T6P precursors when applied 1 d before rewatering after a 9-d drought period (Griffiths et al., 2016). This suggests that T6P is a general stimulator of biosynthetic pathways that are attenuated in a tissue-dependent manner, including starch synthesis in the wheat endosperm and other biosynthetic pathways, including cell walls in vegetative tissue that are required for regrowth. This can be explained through the wide-ranging repressive effects of SnRK1 on biosynthetic pathways (Baena-González et al., 2007) that are derepressed by T6P (Zhang et al., 2009). In addition to pushing physiological boundaries for yield and recovery from drought, chemical intervention provides other advantages. Whereas promoter gene constructs are fixed, chemical applications are flexible. A T6P spray can be applied at particular developmental stages and environmental conditions. This chemical spray could potentially be used for a range of crops. It would need to be tested to ensure that there are no adverse effects, for example, on the susceptibility to pathogens, since sugar signaling is known to play an important role in plant-pathogen interactions (Morkunas and Ratajczak, 2014). However, increasing T6P and trehalose contents may actually be beneficial in this regard (Tayeh et al., 2014). There are other chemicals that can be used in the chemical intervention of plant growth, particularly plant hormones such as auxin, GA, ethylene, and compounds that can oppose the effects of the hormones (Santner et al., 2009). However, T6P is the only growth regulator known that directly targets sugar signaling, Suc use, and Suc allocation.

OVERALL EMERGINGING MODEL FOR MODIFYING T6P FOR CROP IMPROVEMENT

Drawing on the significant yield improvements achieved in maize carrying *MADS6-TPP* (Nuccio et al., 2015; Oszvald et al., 2018) and chemical intervention in wheat (Griffiths et al., 2016), a strategy can be proposed for targeting T6P to reproductive tissue to improve yield. Decreasing T6P in the phloem vasculature that supplies the developing grain with Suc would increase Suc import into the grain by up-regulating *SWEET* gene expression. Increasing T6P in the grain endosperm itself would promote starch biosynthesis within the grain, according to the example with T6P precursors in wheat (Griffiths et al., 2016; Fig. 4). Additionally, selection of *TPS* and *TPP* genes has occurred during maize domestication (Hufford et al., 2012; Table I). Investigating the roles of these genes in other cereals may be particularly promising moving forward. Manipulating genes such as rice *TPP7* may confer better performance in different environments such as flooded rice fields (Kretzschmar et al., 2015). Orphan crops may be particularly amenable to improvement of the trehalose pathway, given the domestication improvements seen in maize. Genetic and chemical interventions can be used as complementary methods to target T6P. As a central regulator of source sink whose optimization and synchrony is essential for the efficiency of carbon generation and utilization for crop yields, T6P is likely to feature strongly in future crop yield and resilience improvement programs. [AU : 6]

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Domain structure of a complete plant *TPS* protein (using AtTPS1 as a model).

Supplemental Figure S2. Domain structure of a complete plant *TPP* protein (using AtTPPA as a model).

Supplemental Table S1. Constitutive domains of *TPS* proteins.

Supplemental Table S2. Constitutive domains of *TPP* proteins.

Supplemental Table S3. Summary of the structural information of the *TPS* genes and proteins.

Supplemental Table S4. Summary of the structural information of the *TPP* genes and proteins.

Supplemental Table S5. Transformation of the wheat TGACv1 *TPS* transcript identifiers into IWGSC RefSeq v1.0 identifiers using tera-blastp.

Supplemental Table S6. Transformation of the wheat TGACv1 *TPP* transcript identifiers into IWGSC RefSeq v1.0 identifiers using tera-blastp.

Supplemental Table S7. Functional annotation of the identified wheat *TPS* proteins according to the IWGSC RefSeq 1.0 reference.

Supplemental Table S8. Functional annotation of the identified wheat *TPP* proteins according to the IWGSC RefSeq 1.0 reference.

Supplemental Table S9. Chromosomal coordinates of the wheat *TPS* genes (in gff3 format) plus structural information.

Supplemental Table S10. Chromosomal coordinates of the wheat *TPP* genes (in gff3 format) plus structural information.

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