Trehalose 6-phosphate promotes seed filling by activating auxin biosynthesis

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Summary

- Plants undergo several developmental transitions during their life cycle. One of these, the differentiation of the young embryo from a meristem-like structure into a highly specialized storage organ, is believed to be controlled by local connections between sugars and hormonal response systems. However, we know little about the regulatory networks underpinning the sugar–hormone interactions in developing seeds.
- By modulating the trehalose 6-phosphate (T6P) content in growing embryos of garden pea (Pisum sativum), we investigate here the role of this signaling sugar during the seed-filling process.
- Seeds deficient in T6P are compromised in size and starch production, resembling the wrinkled seeds studied by Gregor Mendel. We show also that T6P exerts these effects by stimulating the biosynthesis of the pivotal plant hormone, auxin. We found that T6P promotes the expression of the auxin biosynthesis gene TRYPTOPHAN AMINOTRANSFERASE RELATED2 (TAR2), and the resulting effect on auxin concentrations is required to mediate the T6P-induced activation of storage processes.
- Our results suggest that auxin acts downstream of T6P to facilitate seed filling, thereby providing a salient example of how a metabolic signal governs the hormonal control of an integral phase transition in a crop plant.

Introduction

The transition from early patterning into seed filling is an important phase change in developing seeds, ensuring seed survival and the nourishment of seedling growth upon germination. For this reason, plants have evolved a regulatory network to control seed filling, and carbohydrates appear to play a pivotal role in this process (Hills, 2004; Weber et al., 2005). Sucrose is thought to have a dual function in developing seeds as a nutrient sugar and as a signal molecule triggering storage-associated gene expression (Weber et al., 1998). Two decades ago, the invertase control hypothesis of seed development was formulated, suggesting that seed coat-borne invertases prevent the onset of storage processes in the early embryo by cleaving the incoming sucrose into hexoses (Weber et al., 1995a). When invertase activity declines, sucrose concentrations begin to rise and seed filling is initiated. Recent evidence from a study on pea indicates that auxin is a key factor in mediating the response to this metabolic switch. Impairment of auxin biosynthesis in the tar2-1 mutant has been shown to specifically affect the seed-filling stage, mainly by curtailing embryo growth and sucrose partitioning into reserve starch (McAdam et al., 2017). However, in spite of the apparent interrelation between carbohydrate metabolism and auxin in seeds, relatively little is known about the regulatory mechanisms underlying this sugar–auxin link.

Trehalose 6-phosphate (T6P), the intermediate of trehalose biosynthesis, has been shown to be an essential signal metabolite in plants, linking growth and development to carbon metabolism (Lunn et al., 2006; Figueroa & Lunn, 2016b). The sucrose–T6P nexus model postulates that T6P acts as a signal of sucrose availability, helping to maintain sucrose concentrations within a range that is appropriate for the developmental stage of the plant (Yadav et al., 2014). The particular importance of T6P for developmental transitions in plants is underlined by a growing number of growth processes known to be affected by directed modulation of T6P concentrations or by mutations of several trehalose biosynthesis genes (Satoh-Nagasawa et al., 2006; Debast et al., 2011; Wahl et al., 2013). Synthesis of trehalose comprises two catalytic steps. In the first step, T6P synthases (TPSs) catalyze the formation of T6P from glucose 6-phosphate and uridine diphosphate glucose (UDPG). T6P is then dephosphorylated by T6P phosphatases (TPPs) to yield trehalose. A striking example with
respect to seed development involves the mutation of the TPS1 gene in Arabidopsis (Blazquez et al., 1998). Loss of TPS1 causes embryo abortion at the point where the embryo transitions from torpedo to early cotyledon stage (Eastmond et al., 2002), while the accumulation of storage proteins and lipids is compromised (Gómez et al., 2006). Based on these findings, it appears that T6P forms an essential part of a signaling network regulating the initiation and establishment of storage processes in seeds.

The small size of Arabidopsis seeds, however, presents practical difficulties in investigating how T6P participates in the regulation of seed filling. Here, we make use of the large size of pea seeds, allowing the easy preparation and compositional analysis of individual embryos. We engineered transgenic pea plants for embryo-specific expression of TPS (otsA) and TPP (otsB) from Escherichia coli, aiming to elevate and deplete T6P content in developing seeds, respectively. Our results provide genetic and biochemical evidence that T6P reports the raising sucrose status in the maturing embryo, leading to a stimulation of embryo growth and reserve starch biosynthesis. Moreover, our findings show that auxin acts as a key mediator of this process.

Materials and Methods

Plant material

Transgenic pea plants were created within the cv ‘Eri’®, previously described for transgenic USP:TAR2 plants (McAdam et al., 2017). The tar2-1 mutant was made in the background of cv ‘Cameor’ (Tivendale et al., 2012), while those carrying nonwild-type (nonWT) alleles at r, rb and rug4® were near isogenic selections made in, respectively, germplasm accessions WL 200, WL 1685 and SIM91 (John Innes Germplasm Collection, Norwich, UK) (Wang & Hedley, 1993). Plants were grown under a 16 h photoperiod provided by artificial light (550 µE m⁻² s⁻¹). The temperature regime was 19°C : 16°C, light : dark. The plants were fertilized once a week with 0.4% Hakaphos® blau 15 + 10 + 15(+2) (Compo Expert, Münster, Germany) starting 4 wk after sowing. Flowers were tagged at the time of pollination, and seeds were harvested around midday according to the number of d after pollination (DAP) that had elapsed. Embryos were excised from two to three seeds per pod, weighed and snap-frozen in liquid nitrogen.

Transgene construction and the production of transgenic plants

To generate transgenic USP:TPS and USP:TPP pea plants, coding sequences of the respective Escherichia coli genes otsA and otsB were PCR-amplified from plasmids harboring the corresponding cDNAs. The oligonucleotides used to attach an XbaI restriction site to each end of the amplified coding sequences are listed in Supporting information Table S1. The amplicons were XbaI restricted and ligated into the USP:pBar binary vector which contains an embryo-specific expression cassette based on the long version of the USP promoter (Zakharov et al., 2004). Selected plasmids were sequenced for validation purposes and then introduced into Agrobacterium tumefaciens strain EHA 105. The generation of transgenic pea plants was performed according to a modified transformation method using sections from embryo axis (Schroeder et al., 1993). For this purpose, embryo axes were excised from germinating pea seeds (3 d after imbibition), sliced longitudinally into five to seven segments with a scalpel blade, and the obtained explants were immersed in a suspension of Agrobacteria. After 2 d of cocultivation on B₃h medium (Brown & Atanassov, 1985), explants were washed with sterile water and transferred to selective P1 medium (Schroeder et al., 1993) containing 10 mg l⁻¹ phosphinothricin (Duchefa, Haarlem, the Netherlands). After 2 wk of callus formation, shoot growth was induced by cultivation on MS4 medium containing Murashige & Skoog macro- and micronutrients (Murashige & Skoog, 1962), B₅ vitamins (Gamborg et al., 1968), 4 mg l⁻¹ 6-benzylaminopurine, 2 mg l⁻¹ naphthalene acetic acid, 0.1 mg l⁻¹ indol-3-yl butyric acid, and 3% (w/v) sucrose supplemented with 10 mg l⁻¹ phosphinothricin. When the developing shoots were about 5 cm long, a substantial gain in plant growth was induced by grafting the shoots onto a WT root stock. The grafted plantlets were potted and maintained in growth chambers, where they subsequently flowered and produced seeds. Insertion and segregation of the transgenes was verified by PCR using oligonucleotides that are listed in Table S1. The allelic status at the TAR2 locus was assessed by previously described PCR-based genotyping (Tivendale et al., 2012).

Determination of sucrose, starch, total carbon and nitrogen

Snap-frozen embryos were ground to powder and lyophilized at −20°C. Mature seeds were pulverized in a ball mill and the powder dried in a desiccator. To measure tissue sucrose and starch contents, the powder was extracted twice in 80% (v/v) ethanol at 60°C and the supernatants pooled and vacuum-evaporated; the residue was dissolved in sterile water. Sucrose contents were determined enzymatically (Heim et al., 1993). The starch retained in the water-insoluble fraction was solubilized in 1 M KOH and gelatinized by incubating for 1 h at 95°C, after which it was neutralized by the addition of 1 M HCl. The starch content was determined as glucose units, following its complete hydrolysis to glucose using amyloglucosidase (Rolletschek et al., 2002). The carbon and nitrogen content of powdered seed tissue was obtained using a Vario Micro Cube elemental analyser (Elementar UK Ltd, Stockport, UK).

Enzyme activity assays

Enzyme activities were determined in growing embryos of three USP:TPS, five USP:TPP, and the corresponding WT lines, each with five biological replicates per time point. The frozen, pulverized tissue was extracted in 5 volumes of 0.1 M MOPS (pH 7.4), 10 mM MgCl2, 1 mM EDTA, 1 mM EGTA and 2 mM dithiothreitol; the resulting homogenates were centrifuged (10 000 g, 4°C, 5 min) and the supernatants held on ice. The extracts were assayed for adenosine diphosphate glucose pyrophosphorylase (AGP) (Weber et al., 1995b) and phosphoglucomutase (PGM)
(Manjunath et al., 1998) activity. TPP activity was determined by following the release of orthophosphate from trehalose 6-phosphate. The reaction mixtures, containing 25 mM HEPES/KOH, pH 7.0, 8 mM MgCl₂, 0.05 mM Triton X-100, 0.5 mM EDTA, 1.25 mM T6P, and 2 µl of crude extract in a total volume of 20 µl, were incubated for 20 min at 32°C and stopped by application of 10 µl of 0.5 M HCl. The yield of orthophosphate was determined by adding 50 µl of 1% (w/v) ammonium molybdate dissolved in 1 M H₂SO₄ and 20 µl of 10% (w/v) ascorbic acid (Ames, 1966). After incubation at 40°C for 40 min, absorbance was immediately measured at 800 nm.

AGP redox activation
The redox activation of the AGP of developing embryos was determined by monitoring the degree of monomerization of small AGP subunits in nonreducing sodium dodecyl sulfate gels (Hendriks et al., 2003).

RNA extraction, cDNA synthesis and transcript profiling
RNA was isolated from the frozen, pulverized tissue using a phenol/chloroform-based extraction method, followed by LiCl precipitation (Miranda et al., 2001). Contaminating genomic DNA was removed by incubating a 20 µg aliquot of the RNA in a 100 µl reaction containing 4 U TURBO DNA-free (Ambion/Life Technologies, Darmstadt, Germany), following the manufacturer’s protocol. After digestion, samples were desalted and concentrated to a volume of 20 µl using Vivaspin 500 centrifugal concentrators with a molecular weight cutoff of 30 000 Da (Sartorius, Goettingen, Germany). The absence of genomic DNA contamination was confirmed by running a quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) assay directed by a primer targeting intron #8 of the pea PHOSPHOLIPASE C (PLC) gene (Table S1). First-strand cDNA was synthesized from 4.5 µg purified RNA using SuperScript III (Invitrogen) primed by oligo-dT, according to the manufacturer’s instructions. The reference sequence was a fragment of the pea ubiquitin-conjugating enzyme gene E2 (PSC34G03; http://apex.ipk-gatersleben.de), whose expression stability in the developing pea seed was evaluated using geNorm software (Vandesompele et al., 2002). Profiling of TAR2, AGPL, AGPS1, and AGPS2 transcripts via qRT-PCR was performed as 10 µl reactions containing 2 µl of each primer (0.5 µM, sequences given in Table S1), 1 µl cDNA (1 µg µl⁻¹) and 5 µl Power SYBR® Green-PCR Master Mix (Applied Biosystems/Life Technologies, Darmstadt, Germany): the amplification regime consisted of a denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s. PCR amplification efficiencies were estimated using a linear regression method implemented in the LinRegPCR program (Ramakers et al., 2003). Relative transcript abundances were calculated using the 2⁻ΔΔCt method (Livak & Schmittgen, 2001). For the qRT-PCR analysis of transgenic embryos, samples were obtained from two biological replicates for each of five USP::TPP and the corresponding WT lines, whereas four biological replicates each were sampled for two USP::TPS and the corresponding WT lines.

Microarray hybridization and analysis
An 8 × 60 K customized pea eArray (ID 045803, Agilent Technologies, Santa Clara, CA, USA) was used to scan the pea embryo transcriptome. Embryos were sampled at both 14 and 22 DAP from two independent USP:TPP transgenic lines (three independent plants per line), along with 14 DAP embryos from six WT plants and 22 DAP embryos from five WT plants. Total RNA extracts were treated with RNase-free DNase and purified using the RNeasy RNA Isolation Kit (Qiagen). A 100 ng aliquot of RNA was used to generate cRNA, which was Cy3-labeled via the Low Input Quick Amp Labeling Kit (Agilent Technologies). The labeling efficiency, amount, as well as the amount and quality of the cRNA synthesized were monitored using an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and a Bioanalyzer 2100 (Agilent Technologies). A 600 ng aliquot of labeled cRNA was used for fragmentation and array loading (Gene Expression Hybridization Kit, Agilent Technologies). Hybridization, scanning, image evaluation, and feature extraction were achieved as described by Pielot et al. (2015). The data were evaluated with the aid of GENESPRING v.12.5 (Agilent Technologies) using default parameters: relative expression values were achieved after log₂ transformation, quantile normalization and baseline transformation to the median of all samples. After performing moderated t-test and false discovery rate correction (Benjamini–Hochberg), only features associated with a signal intensity difference of at least two-fold and statistical difference (P-corrected ≤ 0.05) between the WT and transgenic lines at a given sampling time point were retained. For stringent data evaluation, outliers and transcripts without significant expression (absolute values ≥ 100) were removed, and a high-stringency P cutoff (P-corrected ≤ 0.001) was used to remove random effects. Genes with known annotation were assembled into 43 functional groups (Table S2).

Extraction and quantification of metabolites and phytohormones
Metabolites (including T6P) were extracted from the frozen, pulverized embryos of three USP:TPS and three USP:TPP transgenics, along with their corresponding WT sibling plants (five embryos per genotype at each time point). The content of soluble metabolites was assessed using high-performance anion-exchange LC coupled to tandem MS (LC-MS/MS) (Figueroa et al., 2016a). To estimate the content of T6P in tar2-1 and TAR2 embryos, freeze-dried samples were extracted (Schwender et al., 2015) followed by ion chromatography using Dionex ICS-5000+ HPIC system (Thermo Scientific, Dreieich, Germany), coupled to a QExactive Plus hybrid quadrupol-orbitrap MS (Thermo Scientific) equipped with a heated electrospray ionization probe. Chromatographic separation was performed on Dionex IonPac 2 × 50 mm and 2 × 250 mm AS11-HC4-µm columns equilibrated with 10 mM KOH at 0.35 ml min⁻¹ flow rate and 35°C
column temperature. A linear gradient of 10–100 mM KOH was generated in 28 min followed by 2 min of column equilibration. The MS spectra were acquired using a full-scan range 67–1000 (m/z) in the negative mode at 140 000 resolving power, 200 ms maximum injection time and an automatic gain control target of 1E6 ions. The source settings included 36 arbitrary units for sheath gas flow rate, 5 arbitrary units for auxiliary gas flow rate. The capillary temperature was set to 320°C and S-lens was set to 50. Quantification was performed with external calibration using authenticated standard and TRACKFINDER 4.1 software (Thermo Scientific). Auxins and 4-CL-tryptophan were extracted from developing embryos harvested from three USP:TPP and three USP::TPP transgenic plants, and also from their corresponding sibling WT embryos, and subsequently quantified (three embryos per genotype at each time point) using ultraperformance LC coupled with MS (Tivendale et al., 2012).

Histological and morphological analysis

Seeds were sliced into two pieces and fixed at room temperature overnight in 50% (v/v) ethanol, 5% (v/v) glacial acetic acid and 4% (v/v) formaldehyde. After dehydration by passing through an ethanol series, the samples were embedded in ParaPlast Plus (Sigma-Aldrich). Cross-sections of thickness 15 µm were mounted on poly-L-lysine-treated slides (Sigma-Aldrich) and stained with iodine in order to visualize the starch grains. The specimens were imaged by differential interference contrast microscopy (Zeiss Axioscy.M2), and AXIOVISION (Zeiss) software was used for scaling; cell areas were estimated from digital images using IMAGEJ (/image.nih.gov/ij/); each measurement was based on sections from three biological replicates. Early embryo growth was verified by using a VHX digital microscope (Keyence, Osaka, Japan).

Nuclear magnetic resonance imaging

Magnetic resonance experiments were performed by using a Bruker Avance III HD 400 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a 1000 mT m−1 gradient system. For the measurement of the transverse relaxation time (T2) in embryonic tissues, excised pea embryos were placed in a saddle coil with an inner diameter of 10 mm. A standard multi-slice multi-echo sequence was applied in three dimensions (3D; repetition time, 2500 ms; echo time, 5 ms; number of echoes, 12). The images were acquired with a resolution of 80 x 90 x 90 µm. The resulting datasets were processed by using the MATLAB software (MathWorks, Natick, MA, USA) with an algorithm written in-house. Calculation of the 3D T2 maps is based on a least-squares algorithm, and the resulting T2 maps were subsequently exported to AMIRA (FEI Visualization Sciences Group, Mérignac, France) for image processing.

Data availability

Microarray data reported in this study have been deposited in the ArrayExpress database at EMBL-EBI (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-6659.

Results

Cotyledon differentiation and starch accumulation are impaired by embryo-specific expression of TPP

To assess the potential role of T6P in the control of seed filling, we performed an initial metabolite analysis of growing pea embryos, revealing that T6P concentrations increased at the transition phase in parallel with sucrose and remained at high concentrations during the storage phase (Fig. 1a). There was a positive correlation between T6P and sucrose (Pearson correlation coefficient, r = 0.84), suggesting that T6P might control the phase transition into seed filling in response to sucrose accumulation. Next, we made use of a well-established approach to manipulate T6P concentrations in plants (Schluemme et al., 2003), and reduced the T6P content in developing pea embryos by heterologous expression of a bacterial TPP, encoded by the otsB gene from Escherichia coli. These transgenic USP:TPP lines were derived from a set of 18 independent T1 plants, five of which were used to establish transgene homozygotes. The expression of the USP:TPP transgene was confirmed by quantifying the activity of TPP and the content of T6P in the developing embryos of these lines. While no TPP activity was detectable in WT embryos, considerable activity was present in USP:TPP embryos (Table S3). This led to a significant depletion of the T6P content in the transgenic embryos (Table S3), resulting in much smaller seeds and a wrinkled seed phenotype at maturity (Fig. 1b; Table S4). Mendel’s wrinkled-seed trait is associated with impaired reserve starch synthesis (Bhattacharyya et al., 1993), as was also the case for the seeds set by USP:TPP plants (Fig. 1c), which contained 50% less starch on a per-seed basis (Table S4). At the same time, sucrose concentrations were elevated in transgenic embryos (Fig. 1d), indicating that the sucrose-to-starch conversion was affected. Microscopic examination supported this finding, with USP:TPP embryos harboring considerably fewer and smaller starch granules than WT embryos (Fig. 1e). However, altered starch accumulation only partially explains the diminished DW of USP:TPP seeds (Table S4). Reduced cotyledon growth and smaller cell size also contribute to the decrease in DW (Fig. 1f; Table S5). These effects acted together to compromise the increase in embryo FW in the late stages of development (Fig. 1g), while early embryo growth and organ determinacy were unaffected by the presence of the transgene (Fig. S1). Nuclear magnetic resonance imaging of USP:TPP cotyledons revealed a substantial impairment in the formation of a spatial gradient in T2 transverse relaxation time (Fig. 1h). The differences between the T2 signal of WT and transgenic embryos are mainly a result of reduced enlargement of starch granules as well as increasing vacuolization towards the abaxial (inner) parts of the differentiating USP:TPP cotyledons (Van As, 2006; Borisjuk et al., 2012). Altogether, altered differentiation of USP:TPP embryos is in good agreement with defective cotyledon growth in the Arabidopsis tps1 mutant (Eastmond et al., 2002; Gomez et al., 2006). These results indicate that T6P is a key factor in mediating the phase transition from patterning into seed filling, with at least two major processes being influenced by T6P: the
conversion of sucrose into reserve starch, and the gradual differentiation of cotyledons.

T6P promotes sucrose-to-starch conversion by activating key enzymes of starch synthesis at the transcript level

Our current understanding of starch biosynthesis in pea seeds derives from extensive studies on mutations at different *rugosus* (r) loci, which curtail the activity of individual starch enzymes (Fig. S2a). In an attempt to identify the enzymatic steps within the sucrose-to-starch conversion process that were regulated by T6P, we compared the metabolic changes in *USP:TPP* embryos with those elicited by *r*, *rb*, and *rug4* mutations (Bhattacharyya *et al.*, 1990; Hylton & Smith, 1992; Craig *et al.*, 1999). In transgenic embryos, concentrations of hexose phosphates and UDPG were consistently elevated (Fig. S2b), while only adenosine diphosphate glucose (ADPG) concentrations were markedly lower than in the WT (Fig. 2a). A similar result was obtained only in the developing *rb* embryos (Table S6), which have impaired ADPG-pyrophosphorylase (AGP) activity (Hylton &
Transcriptional changes detected by microarray

To identify global changes in the transcriptome, we performed a microarray analysis of *USP:TPP* embryos harvested at either the transition (14 DAP) or main storage phase (22 DAP). Compared with the WT, a total of 995 (279 decreased, 716 increased) and 2656 (844 decreased, 1812 increased) genes were deregulated at 14 and 22 DAP, respectively (Table S7). From those, transcripts associated with starch biosynthesis were repressed, and the two small subunit encoding genes *AGPS1* and *AGPS2* were significantly repressed in *USP:TPP* embryos (Fig. 2c). The strongest reduction by up to 70% was recorded for *AGPL*, the transcription of which corresponds to the measured changes in AGP activity (Fig. 2b) and tends to peak during the period when the pea seed is most rapidly accumulating starch (Burgess et al., 1997). The loss of AGPL is the underlying cause for seed wrinkling in *rb* mutants (Hylton & Smith, 1992), suggesting that the wrinkled phenotype observed in *USP:TPP* seeds is mainly a result of a reduction in AGPL expression. The implication of these results is that T6P controls the conversion of sucrose into starch, at least in part, by modulating AGP activity at the transcript level.

Auxin acts downstream of T6P to facilitate seed filling

To uncover novel signaling components that mediate the effects of T6P on seed filling, we performed stringent data filtering of the microarray experiment. This analysis identified *TAR2*, the expression of which was unique in being dramatically reduced at both 14 and 22 DAP, as a possible target of T6P (Table S8). The repression of *TAR2* in *USP:TPP* embryos was verified by qRT-PCR (Fig. 3a). In pea, TAR2 participates in auxin biosynthesis
via the indole-3-pyruvic acid pathway, and mutation of the correspond-
ingene leads to reduced concentrations of 4-chloro-indole-3-acetic acid (4-Cl-IAA), the predominant auxin in maturing seeds (Tivendale et al., 2012). Remarkably, the tar2-1 mutation affects the phase transition into seed filling, resulting in the formation of small, wrinkled seeds with decreased starch content and a considerably lower level of AGP activity (McAdam et al., 2017). Our finding that USP:TPP seeds phenocopied those of the tar2-1 mutant, and that introduction of USP:TPP into a tar2-1 background had no additional phenotypic effects beyond those of the parental lines (Fig. 3b; Table 1, Expt 1), raises the possibility that both T6P and TAR2 act in the same signaling pathway. Measurement of the auxin content of USP:TPP embryos revealed a notable decrease in that of 4-Cl-IAA, by up to 70% (Fig. 3c), while at the same time the content of the TAR2-specific substrate, 4-Cl-tryptophan, was higher than in WT embryos (Fig. 3d). Together with the considerable increase of T6P in tar2-1 embryos (Fig. 3e), these results provide evidence that T6P acts as an upstream regulator of TAR2. As proof of this, we created hybrids between USP:TPP plants and transgenic plant lines harboring the USP:TAR2 transgene, which directs expression of the TAR2 coding sequence under the control of the embryo-specific USP promoter. To generate combinations of both transgenes, two previously generated USP:TAR2 lines, #3 and #5 (McAdam et al., 2017), were crossed with USP:TPP #2 and #3 plants (Table 1, Expt 2). Segregants from these crosses were used to establish three double transgene homozygotes, referred to as USP:TPP #2/USP:TAR2 #3, USP:TPP #3/USP:TAR2 #3 and USP:TPP #3/USP:TAR2 #5. The activity of the USP:TAR2 transgene was able to largely restore seed size and starch content to WT values (Fig. 3f; Table 1, Expt 2), even though the level of TPP activity was still considerable (Table S9). Regardless of these reconstituting effects, embryos formed by the homozygous USP:TPP/USP:TAR2 plants shared the same pale green color as those formed by USP:TPP plants (Fig. 3g), indicating that T6P regulates developmental processes in addition to those involving the TAR2 pathway. Taken together, our findings strongly suggest that normal cotyledon growth and reserve starch accumulation are both dependent on the transcriptional activation of TAR2 by T6P.

Embryo-specific elevation of T6P induces auxin and starch biosynthesis

We next investigated the effect of elevated T6P on seed-filling processes by heterologously expressing otaA, an Escherichia coli gene encoding TPS, in an embryo-specific manner. To this end, five homozygous USP:TPS lines were generated from a set of 22 independent T1 plants. Analysis of developing embryos harvested from three of these lines showed that they contained considerably more T6P than those of their sibling WT embryos (Table S10), confirming the functional expression of the bacterial TPS.
Consistent with similar experiments conducted in both Arabidopsis and potato (Schlüpmann et al., 2003; Debast et al., 2011; Yadav et al., 2014), the activity of the transgene resulted in a substantial depletion in the content of soluble sugars (Fig. S4).

Short-term, induced elevation of T6P in Arabidopsis has been shown to induce a loss in sucrose content, owing to a shift in assimilate partitioning away from sucrose in favor of organic and amino acids in the light, or inhibition of transitory starch turnover in the dark (Martins et al., 2013; Figueroa et al., 2016a).

The exposure of wheat plants to cell-permeable forms of T6P has been shown to promote the size and starch content of the grain and to raise AGP activity (Griffiths et al., 2016), an observation that ties in with the increased AGP activity (Fig. 4a) in TPS-expressing pea embryos. Compared with the WT, expression of TAR2 was induced in USP:TPP expressing embryos (Fig. 4b) accompanied by an increase in 4-Cl-IAA concentrations at later stages (Fig. 4c). It appears that the elevation of T6P has a positive influence on the sucrose-to-starch conversion by inducing AGP, and we conclude that this is mediated by a prolonged stimulation of auxin synthesis via TAR2. Despite these favorable changes, neither the starch content nor the size of USP:TPP seeds was affected (Table S11). This is not that surprising, considering that the limits to the final size of the embryo and its capacity to accumulate dry matter are largely influenced by the maternal
of evidence showing interaction between the signaling sugar T6P and the major plant hormone auxin, as a requisite for normal seed filling in pea. We showed that T6P and sucrose concentrations increased in parallel at the time point when the embryo starts to build up storage products, and by manipulating the T6P content in embryos, we found that the transition into the storage mode is based on this relationship. Our results imply that T6P regulates seed filling by promoting cell differentiation and starch accumulation in the maturing embryo, thereby allowing efficient utilization of incoming sucrose. This finding is in agreement with the T6P–sucrose nexus (Yadav et al., 2014) and complements the existing view on the dual function of sucrose as a key metabolite and signaling molecule of seed filling (Hills, 2004; Weber et al., 2005), with T6P reporting the change in sucrose concentration to the regulatory network of embryo differentiation. As in most sink organs, maturing embryos receive sucrose from the phloem and its cleavage is the initial step in the direction of storage product synthesis. However, sucrose is also required to induce storage-related gene expression causing upregulation of important enzymes like AGP (Müller-Röber et al., 1990; Weber et al., 1998). Furthermore, cell expansion in explanted *Vicia faba* embryos is triggered in response to sucrose feeding (Weber et al., 1996). The evidence presented here clearly indicates that most effects that have previously been ascribed to a signaling function of sucrose are principally controlled via a T6P-mediated pathway. We suggest that T6P connects the sucrose state with other regulatory components involved in the control of storage metabolism and embryo differentiation, such as SnF1-related protein kinase1 (Radcuk et al., 2006). This energy sensor coordinates metabolic and hormonal signals with embryo growth (Radcuk et al., 2010). In developing tissues, SnRK1 activity is inhibited by T6P in the presence of an as yet uncharacterized protein (Zhang et al., 2009), and binding of T6P to the catalytic subunit (SnRK1α1) disrupts association and activation of SnRK1 by the SnRK1 activating kinase (SnAK)/Rep-Interacting Kinase1 (GRIK) protein kinases (Zhai et al., 2018).

**Discussion**

Efficient deposition of storage compounds in seeds is a key determinant of crop yield. The interplay between carbohydrates and hormones seems to play a crucial role in the control of seed filling, but the underlying regulatory network of this process remains undefined. In this study, we provide several lines of evidence showing interaction between the signaling sugar T6P and the major plant hormone auxin, as a requisite for normal seed filling in pea. We showed that T6P and sucrose concentrations increased in parallel at the time point when the embryo starts to build up storage products, and by manipulating the T6P content in embryos, we found that the transition into the storage mode is based on this relationship. Our results imply that T6P regulates seed filling by promoting cell differentiation and starch accumulation in the maturing embryo, thereby allowing efficient utilization of incoming sucrose. This finding is in agreement with the T6P–sucrose nexus (Yadav et al., 2014) and complements the existing view on the dual function of sucrose as a key metabolite and signaling molecule of seed filling (Hills, 2004; Weber et al., 2005), with T6P reporting the change in sucrose concentration to the regulatory network of embryo differentiation. As in most sink organs, maturing embryos receive sucrose from the phloem and its cleavage is the initial step in the direction of storage product synthesis. However, sucrose is also required to induce storage-related gene expression causing upregulation of important enzymes like AGP (Müller-Röber et al., 1990; Weber et al., 1998). Furthermore, cell expansion in explanted *Vicia faba* embryos is triggered in response to sucrose feeding (Weber et al., 1996). The evidence presented here clearly indicates that most effects that have previously been ascribed to a signaling function of sucrose are principally controlled via a T6P-mediated pathway. We suggest that T6P connects the sucrose state with other regulatory components involved in the control of storage metabolism and embryo differentiation, such as SnF1-related protein kinase1 (Radcuk et al., 2006). This energy sensor coordinates metabolic and hormonal signals with embryo growth (Radcuk et al., 2010). In developing tissues, SnRK1 activity is inhibited by T6P in the presence of an as yet uncharacterized protein (Zhang et al., 2009), and binding of T6P to the catalytic subunit (SnRK1α1) disrupts association and activation of SnRK1 by the SnRK1 activating kinase (SnAK)/Rep-Interacting Kinase1 (GRIK) protein kinases (Zhai et al., 2018).

**Table 1** The effect on seed weight and starch content of introducing either the tar2-1 mutant allele (Expt 1) or the *USP:TAR2* transgene (Expt 2) into *USP:TPP* plants.

|                      | Starch (mg g⁻¹ DW) | Seed weight (mg) |
|----------------------|-------------------|-----------------|
| Expt 1               |                   |                 |
| TAR2                 | 438 ± 8 a         | 335 ± 23 a      |
| tar2-1               | 311 ± 16 b        | 155 ± 10 c      |
| Empty vector/TAR2    | 422 ± 15 a        | 323 ± 11 a      |
| Empty vector/tar2-1  | 340 ± 17 b        | 129 ± 9 c       |
| *USP:TPP #1/TAR2     | 356 ± 10 b        | 212 ± 7 b       |
| *USP:TPP #1/tar2-1   | 348 ± 3 b         | 148 ± 7 c       |
| TAR2 (Cameor × Erbi) | 433 ± 12 a        | 313 ± 10 a      |
| tar2-1 (Cameor × Erbi)| 336 ± 13 b       | 139 ± 7 c       |
| *USP:TPP #2/TAR2     | 350 ± 10 b        | 203 ± 11 b      |
| *USP:TPP #2/tar2-1   | 349 ± 9 b         | 151 ± 6 c       |
| TAR2 (Cameor × Erbi) | 429 ± 10 a        | 312 ± 8 a       |
| tar2-1 (Cameor × Erbi)| 342 ± 5 b        | 150 ± 7 c       |
| Expt 2               |                   |                 |
| Empty vector         | 497 ± 12 a        | 298 ± 3 a       |
| *USP:TPP #2          | 397 ± 23 b        | 193 ± 14 b      |
| *USP:TPP #3          | 384 ± 8 b         | 199 ± 12 b      |
| *USP:TAR2 #3         | 466 ± 22 a        | 320 ± 8 a       |
| *USP:TAR2 #5         | 521 ± 23 a        | 306 ± 6 a       |
| *USP:TPP #2/USP:TAR2 #3 | 507 ± 13 a    | 306 ± 18 a      |
| *USP:TPP #3/USP:TAR2 #3 | 482 ± 21 a    | 277 ± 5 a       |
| *USP:TPP #3/USP:TAR2 #5 | 497 ± 30 a   | 312 ± 7 a       |

Values are means ± SEM (*n* = 5). Means labeled with the same letter (a–c) are not statistically significantly different from one another (*P* ≤ 0.01).

**Fig. 4** Expression of trehalose 6-phosphate synthase (TPS) induces starch and auxin synthesis in developing pea embryos. (a) The level of adenosine diphosphate glucose-pyrophosphorylase (AGP) activity in 18- to 26-d-old USP:TPS and wild-type (WT) embryos (*n* = 15): *, *P* ≤ 0.05 (Student’s t-test). (b) Relative transcript abundances of TAR2 in embryos formed by USP:TPS and corresponding WT plants. Transcript abundances are means ± SEM (*n* = 8): *, *P* ≤ 0.05; **, *P* ≤ 0.01 (Student’s t-test). (c) Auxin accumulation in growing USP:TPS and WT embryos. Values are means ± SEM (*n* = 9): *, *P* ≤ 0.05 (Student’s t-test); ns, not significant.
Until now, the underlying mechanism by which T6P integrates carbohydrate partitioning with the hormonal control of plant development has not been apparent. Importantly, our data now indicate that auxin is a key factor in mediating the effects of T6P, which acts upstream of the pivotal auxin biosynthesis gene TAr2 (McAdam et al., 2017) to trigger seed filling in pea. We propose that this process is mediated via a modulation of the auxin 4-Cl-IAA (Fig. 5), the concentration of which increases sharply at the transition stage (Tivendale et al., 2012). There is a growing body of evidence that soluble sugars control plant growth by modifying auxin biosynthesis (LeClere et al., 2010; Sairanen et al., 2012; Lilley et al., 2012; Barbier et al., 2015). Altered sugar concentrations in endosperm-defective miniature1 kernels of maize have been suggested to induce auxin deficiency as a result of the suppression of the genes ZmTAR1 and ZmYUCCA1 (LeClere et al., 2010). Both of these genes encode proteins involved in the indole 3-pyruvic acid branch of auxin biosynthesis (Won et al., 2011; Stepanova et al., 2011), with ZmYUCCA1 being essential for the formation of a normal endosperm (Bernardi et al., 2012). Apart from seeds, a similar connection between sugars and auxin has been implicated in the control of shoot branching. Contradicting the classical theory of apical dominance (Thimann et al., 1934), the accumulation of sucrose enables the initiation of bud outgrowth after decapitation in pea (Mason et al., 2014), an effect thought to be mediated by T6P (Fichtner et al., 2017). Notably, feeding of sucrose stimulates auxin synthesis within buds and promotes sustained auxin export from bud to stem (Barbier et al., 2015). Collectively, our findings indicate that the hitherto unknown interaction between T6P and auxin might play a general role in mediating the sugar–auxin link. It will be of ongoing interest to determine how this relationship fits within the current understanding of the regulatory frameworks surrounding growth processes and developmental transitions in plants.

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Author contributions

TM directed the overall study design and performed enzyme activity measurements, microscopy, transcript profiling, compositional analysis of seeds, molecular cloning, plant transformation, cross-breeding and genotyping. AH, RF and JEL conducted the LC-MS-based profiling of metabolites. IT and PG analyzed the redox status of AGP. Microarray experiments were performed and analyzed by RR. ELM and JJR performed the measurement of hormones. Nuclear magnetic resonance images were generated by EM and LB. TM wrote the paper, on which all authors commented.

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References

Ames BN. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. Methods in Enzymology 8: 115–118.

Barbier F, Péron T, Lecerc M, Pérez-García M-D, Barrière Q, Rolick J, Boutet-Mercey S, Citérner S, Lemoine R, Porcheron B et al. 2015. Sucrose is an early modulator of the key hormonal mechanisms controlling bud outgrowth in Rosa hybrida. Journal of Experimental Botany 66: 2560–2582.

Bardanti J, Lanubile A, Li Q-B, Kumar D, Kladnik A, Cook SD, Ross JJ, Marocco A, Chourey PS. 2012. Impaired auxin biosynthesis in the defective endosperm18 mutant is due to mutational loss of expression in the ZmYuc1 synthase 1, which catalyses the first step in trehalose synthesis, is essential for Arabidopsis embryo maturation. The Plant Journal 29: 225–235.

Fichtner F, Barbier FF, Feil R, Watanabe M, Annunziata MG, Chabikwa TG, Höffgen R, Stitt M, Beveridge CA; Lunn JE. 2006. Trehalose-6-phosphate synthase 1, which catalyses the first step in trehalose synthesis, is essential for Arabidopsis embryo maturation. The Plant Journal 29: 225–235.

Figueroa CM, Feil R, Ishihara H, Watanabe M, Kölling K, Krause U, Höhne M, Encke B, Plaxton WC, Zeeman SC et al. 2016a. Trehalose-6-phosphate coordinates organic and amino acid metabolism with carbon availability. The Plant Journal 85: 410–423.

Figueroa CM, Lunn JE. 2016b. A tale of two sugars: trehalose-6-phosphate and sucrose. Plant Physiology 172: 7–27.

Gamborg OL, Miller R, Ojima K. 1968. Nutrient requirements of suspension cultures of soybean root cells. Experimental Cell Research 50: 151–158.

Gómez LD, Baud S, Gilday A, Li Y, Graham IA. 2006. Delayed embryo development in the ARABIDOPSIS Trehalose-6-phosphate Synthase 1 mutant is associated with altered cell wall structure, decreased cell division and starch accumulation. The Plant Journal 46: 69–84.

Griffiths CA, Sagar R, Primavesi LF, Patel MK, Passarelli MK, Gilmore IS, Steven RT, Bunch J, Paul MJ. 2016. Chemical intervention in plant sugar signalling increases yield and resilience. Nature 540: 574–578.

Heim U, Weber H, Bäumlein H, Wobus U. 1993. A sucrose-synthase gene of Vicia faba L.: expression pattern in developing seeds in relation to starch synthesis and metabolic regulation. Planta 191: 394–401.

Hendriks JH, Kolbe A, Gibon Y, Stitt M, Geigenberger P. 2003. ADP-glucose pyrophosphorylase is activated by posttranslational redox-modification in response to light and to sugars in leaves of Arabidopsis and other plant species. Plant Physiology 133: 838–849.

Hills MJ. 2004. Control of storage-product synthesis in seeds. Current Opinion in Plant Biology 7: 302–308.

Hylton C, Smith AM. 1992. The rbr mutation of peas causes structural and regulatory changes in ADP glucose pyrophosphorylase from developing embryos. Plant Physiology 99: 1626–1634.

LeClere S, Schmelz EA, Chourey PS. 2010. Sugar levels regulate tryptophan-dependent auxin biosynthesis in developing maize kernels. Plant Physiology 153: 306–318.

Leyser O. 2018. Auxin signaling. Plant physiology 176: 465–479.

Lilley JL, Gee CW, Sairanen I, Ljung K, Nemhauser JL. 2012. An endogenous carbon-sensing pathway triggers increased auxin flux and hypocotyl elongation. Plant Physiology 160: 2261–2270.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. Methods 25: 402–408.

Lunn JE, Feil R, Hendriks JHM, Gibon Y, Morcuende R, Osuna D, Scheible WR, Carillo P, Hajirezaei MR, 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.

Majda M, Robert S. 2018. The role of auxin in cell wall expansion. International Journal of Molecular Sciences 19: 951.
Manjunath S, Lee CHK, Van Winkle P, Bailey-Serres J. 1998. Molecular and biochemical characterization of cytosolic phosphoglucomutase in maize: expression during development and in response to oxygen deprivation. Plant Physiology 117: 997–1006.

Martins MCM, Heizari M, Fettke J, Steup M, Feil R, Krause U, Arrivault S, Vosloh D, Figueroa CM, Ivakov A et al. 2013. Feedback inhibition of starch degradation in Arabidopsis leaves mediated by trehalose-6-phosphate. Plant Physiology 163: 1142–1163.

McAdam EL, Meitzel T, Quiettenden LJ, Davidson SE, Dalmais M, Bendahmane Al, Thomson R, Smith JJ, Nichols DS, Urquhart S et al. 2017. Evidence that auxin is required for normal seed size and starch synthesis in pea. New Phytologist 216: 193–204.

Miranda M, Borisjuk L, Tewes A, Heim U, Sauer N, Wobus U, Weber H. 2001. Amino acid permeases in developing seeds of Vicia faba L.: expression precedes storage protein synthesis and is regulated by amino acid supply. The Plant Journal 28: 61–71.

Müller-Röber BT, Koßmann J, Hannah LC, Willmitzer L, Sonnewald U. 1990. One of two different ADP-glucose pyrophosphorylase genes from potato responds strongly to elevated levels of sucrose. Molecular Genetics and Genomics 224: 136–146.

Muñoz-Nortes T, Pérez-Pérez JM, Sarmiento-Manís R, Candela H, Micol JL. 2017. Deficient glutamate biosynthesis triggers a concerted upregulation of cytosomal protein genes in Arabidopsis. Scientific Reports 7: 1–14.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15: 473–497.

Paul MJ, Jhurreea D, Zhang Y, Primavesi LF, Delatte T, Schluemmehn H, Wingler A. 2010. Up-regulation of biosynthetic processes associated with growth by trehalose-6-phosphate. Plant signaling & behaviour 5: 386–392.

Pelot R, Kohl S, Manz B, Rutten T, Weier D, Tarkowski D, Rolcik J, Strnad M, Volke F, Weber H. 2015. Hormone-mediated growth dynamics of the barley pericarp as revealed by magnetic resonance imaging and transcript analysis of quantitative real-time polymerase chain reaction (PCR) data. Journal of Experimental Botany 66: 6927–6943.

Radchuk R, Emery RJN, Weier D, Vigeolas H, Geigenberger P, Lunn JE, Feil R, Weschke W, Weber H. 2010. Sucrose non-fermenting kinase 1 (SnRK1) coordinates metabolic and hormonal signals during pea cotyledon growth and differentiation. The Plant Journal 61: 324–338.

Radchuk R, Radchuk V, Weschke W, Borisjuk L, Weber H. 2006. Repressing the expression of the SUCROSE NONFERMENTERING-1-RELATED PROTEIN KINASE gene in pea embryo causes pleiotropic defects of maturation similar to an abscisic acid-insensitive phenotype. Plant Physiology 140: 263–278.

Ramakers C, Ruijter JM, Depluiz RH, Moorman AF. 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neuroscience Letters 339: 62–66.

Rolletschek H, Hajirezaei M-R, Wobus M, Weber H. 2002. Antisense-inhibition of ADP-glucose pyrophosphorylase in Vicia narbonensis seeds increases soluble sugars and leads to higher water and nitrogen uptake. Planta 214: 954–964.

Sairanen I, Novák O, Pencík A, Ikeda Y, Jones B, Sandberg G, Ljung K. 2012. Soluble carbohydrates regulate auxin biosynthesis via PIF proteins in Arabidopsis. Plant Cell 24: 4907–4916.

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.

Schluemmehn H, Pellny T, van Dijken A, Smeekens S, Paul M. 2003. Trehalose-6-phosphate is indispensable for carbohydrate utilization and growth in Arabidopsis thaliana. Proceedings of the National Academy of Sciences, USA 100: 6849–6854.

Schoedler HE, Schotz AH, Wardley-Richardson T, Spencer D, Higgins TJ. 1993. Transformation and regeneration of two cultivars of pea (Pisum sativum L.). Plant Physiology 101: 751–757.

Schwender J, Hebbelmann I, Heinzel N, Hildebrandt T, Rogers A, Naik D, Klapperstuck M, Braun H-P, Schreiber F, Denolf P et al. 2015. Quantitative multilevel analysis of central metabolism in developing oilseeds of oilseed rape during in vitro culture. Plant Physiology 168: 828–848.

Stepanova AN, Yun J, Robles LM, Novak O, He W, Guo H, Ljung K, Alonso JM. 2011. The Arabidopsis YUCCA1 flavin monooxygenase functions in the indole-3-pyruvic acid branch of auxin biosynthesis. Plant Cell 23: 3961–3973.

Thimann KV, Skoog F, Kerkhoff WG. 1934. On the inhibition of bud development and other functions of growth substance in Vicia faba. Proceedings of the Royal Society B: Biological Sciences 114: 317–339.

Tivendale ND, Davidson SE, Davies NW, Smith JA, Dalmais M, Bendahmane AI, Quiettenden LJ, Sutton L, Bala RK, Le Signor C et al. 2012. Biosynthesis of the halogenated auxin, 4-chloroindole-3-acetic acid. Plant Physiology 159: 1055–1063.

Van As H. 2006. Intact plant MRI for the study of cell wall relations, membrane permeability, cell-to-cell and long distance water transport. Journal of Experimental Botany 58: 743–756.

Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe 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.

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: 704–707.

Wang TL, Hedley CL. 1993. Seed mutants in Pisum. Pisum Genetics 25: 64–70.

Weber H, Borisjuk L, Heim U, Buchner P, Wobus U. 1995a. Seed coat-associated invertases of fava bean control both unloading and storage functions: cloning of cDNAs and cell type-specific expression. Plant Cell 7: 1835–1846.

Weber H, Borisjuk L, Wobus U. 1996. Controlling seed development and seed size in Vicia faba: a role for seed coat-associated invertases and carbohydrates state. The Plant Journal 10: 823–834.

Weber H, Borisjuk L, Wobus U. 2005. Molecular physiology of legume seed development. Annual Review of Plant Biology 56: 253–279.

Weber H, Heim U, Borisjuk L, Wobus U. 1995b. Cell-type specific, coordinate expression of two ADP-glucose pyrophosphorylase genes in relation to starch biosynthesis during seed development of Vicia faba L. Planta 195: 352–361.

Weber H, Heim U, Golomboek S, Borisjuk L, Manteuffel U, Wobus U. 1998. Expression of a yeast-derived invertase in developing cotyledons of Vicia narbonensis alters the carbohydrate state and affects storage functions. The Plant Journal 16: 163–172.

Weigt K, Küster H, Rutten T, Fait A, Fernie AR, Miersch O, Wasternack C, Emery RJ, DESel C, Hosien F et al. 2009. ADP-glucose pyrophosphorylase-deficient pea embryos reveal specific transcriptional and metabolic changes of carbon-nitrogen metabolism and stress responses. Plant Physiology 149: 395–411.

Won C, Shen X, Mashiguchi K, Zheng Z, Dai X, Cheng Y, Kasahara H, Kamiya Y, Chorey J, Zhao Y. 2011. Conversion of tryptophan to indole-3-acetic acid by TRYPTOPHAN AMINOTRANSFERASES of Arabidopsis and Yucca in Arabidopsis. Proceedings of the National Academy of Sciences, USA 108: 18518–18523.

Yadav UP, Ivakov A, Feil R, Duan GY, Walthier D, Giavalisco P, Piques M, Carillo P, Hubberten H-M, Stitt M et al. 2014. The sucrose–trehalose 6-phosphate (Tre6P) nexus: specificity and mechanisms of sucrose signalling by Tre6P. Journal of Experimental Botany 65: 1051–1068.

Zalharov A, Giersberg M, Hosein F, Melzer M, Müntz K, Saalbach I. 2004. Seed-specific promoters direct gene expression in non-seed tissue. Journal of Experimental Botany 55: 1463–1471.

Zhai Z, Keeretaweep J, Liu H, Feil R, Lunn JE, Shanklin J. 2018. Trehalose-6-phosphate positively regulates fatty acid synthesis by stabilizing WRINKLED1. Plant Cell 30: 2616–2627.

Zhang Y, Primavesi LF, Jhureeza D, Andralojc PJ, Mitchell RAC, Powers SJ, Schluemmehn 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.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Early development of USP:TTP embryos.
**Fig. S2** Analysis of phosphorylated intermediates directly involved in the sucrose-to-starch conversion.

**Fig. S3** The effect of heterologous TPP expression on PGM activity.

**Fig. S4** Heterologous expression of TPS in pea embryos affects sucrose and sugar phosphate concentrations.

**Table S1** Oligonucleotide sequences employed.

**Table S2** Differentially expressed genes in 14- and 22-d-old USP:TPP embryos (compared with the WT) as revealed by microarray analysis (see separate file).

**Table S3** TPP activity and T6P content in 16-d-old embryos of five homozygous USP:TPP and their corresponding WT lines.

**Table S4** Compositional analysis of mature seeds harvested from transgenic USP:TPP and corresponding WT plants.

**Table S5** Effect of TPP expression on embryo morphology.

**Table S6** The concentration of soluble sugars in 24 DAP embryos harvested from r, rb and rug4 mutant plants.

**Table S7** The extent of AGP monomerization in 18-, 22-, and 26-d-old DAP embryos, harvested from plants harboring the USP:TPP transgene.

**Table S8** Differentially expressed genes in USP:TPP embryos (compared with the WT) at both 14 and 22 DAP, as revealed by stringent microarray data filtering.

**Table S9** TPP activities in 26-d-old embryos formed by hybrids between USP:TPP and USP:TAR2 plants.

**Table S10** The T6P content of 16-d-old USP:TPS and the corresponding WT embryos.

**Table S11** Compositional analysis of mature seeds harvested from transgenic USP:TPP and corresponding WT plants.

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