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Overexpression of Plastid Transketolase in Tobacco Results in a Thiamine Auxotrophic Phenotype[OPEN]

Mahdi Khozeai,[a,1,2] Stuart Fisk,[a,1] Tracy Lawson,[a] Yves Gibon,[b,3] Ronan Sulpice,[b,4] Mark Stitt,[b] Stephane C. Lefebvre,[a,1,5] and Christine A. Raines[a,6]

[a] School of Biological Sciences, University of Essex, Colchester CO4 3SQ, United Kingdom
[b] Max-Planck-Institut für Molekulare Pflanzenphysiologie, 114476 Potsdam-Golm, Germany

To investigate the effect of increased plastid transketolase on photosynthetic capacity and growth, tobacco (*Nicotiana tabacum*) plants with increased levels of transketolase protein were produced. This was achieved using a cassette composed of a full-length *Arabidopsis thaliana* transketolase cDNA under the control of the cauliflower mosaic virus 35S promoter. The results revealed a major and unexpected effect of plastid transketolase overexpression as the transgenic tobacco plants exhibited a slow-growth phenotype and chlorotic phenotype. These phenotypes were complemented by germinating the seeds of transketolase-overexpressing lines in media containing either thiamine pyrophosphate or thiamine. Thiamine levels in the seeds and cotyledons were lower in transketolase-overexpressing lines than in wild-type plants. When transketolase-overexpressing plants were supplemented with thiamine or thiamine pyrophosphate throughout the life cycle, they grew normally and the seed produced from these plants generated plants that did not have a growth or chlorotic phenotype. Our results reveal the crucial importance of the level of transketolase activity to provide the precursor for synthesis of intermediates and to enable plants to produce thiamine and thiamine pyrophosphate for growth and development. The mechanism determining transketolase protein levels remains to be elucidated, but the data presented provide evidence that this may contribute to the complex regulatory mechanisms maintaining thiamine homeostasis in plants.

INTRODUCTION

The Calvin Benson (C3) cycle is the primary pathway of atmospheric CO_2 uptake and fixation into organic molecules. The fixed carbon is used for sucrose and starch biosynthesis and is also essential for biosynthesis of aromatic amino acids and phenylpropanoids in the shikimate pathway and for isoprenoid biosynthesis via the methylerythritol pathway (MEP) (Geiger and Servaites, 1994; Herrmann and Weaver, 1999; Lichtenthaler, 1999). One area of research on the C3 cycle has been to identify enzymes that limit carbon fixation with a view to improving photosynthesis and yield (Raines, 2006; Zhu et al., 2007; Stitt et al., 2010). Using antisense technology it has been shown that sedoheptulose-1,7-bisphosphatase (SBPase), a highly regulated enzyme catalyzing a nonreversible reaction in the regenerative phase of the C3 cycle, limits carbon fixation and plant growth (Harrison et al., 1998; Raines et al., 1999; Harrison et al., 2001; Olçer et al., 2001; Raines, 2003; Lawson et al., 2006; Raines and Paul, 2006). This led to the hypothesis that by increasing the level of this enzyme it might be possible to increase photosynthesis. Overexpression of *Arabidopsis thaliana* SBPase in transgenic tobacco (*Nicotiana tabacum*) provided evidence that increasing the activity of a single native C3 cycle enzyme can result in an increase in carbon fixation and growth (Lefebvre et al., 2005). However, it was also clear from this analysis that overexpression of SBPase did not lead to increased photosynthesis and growth under short-day and low-light conditions (Lefebvre et al., 2005). This work, together with modeling studies, left open the possibility that additional improvement in photosynthesis and yield is likely to be possible through manipulation of additional enzymes of the C3 cycle (Long et al., 2006; Zhu et al., 2007; Raines, 2011).

Plastid transketolase (TK) is another enzyme in the C3 cycle that has been shown to have a significant effect on photosynthesis and growth in antisense tobacco plants (Henkes et al., 2001). Analysis of tobacco plants expressing an antisense construct for plastid TK demonstrated that a small reduction in the activity of this enzyme by 20 to 40% inhibited photosynthesis and significantly decreased the levels of aromatic amino acids and phenylpropanoids (Henkes et al., 2001). Comparison of these results with those from analysis of antisense SBPase and FBPaldolase plants showed that these changes are unique to TK and do not simply reflect a general reduction in the availability of carbon from the C3 cycle (Haake et al., 1998, 1999; Harrison et al., 1998). This effect of TK on carbon allocation may be due to its central location in the C3 cycle where it catalyzes the reversible transfer of a two-carbon molecule to glyceraldehyde 3-phosphate (G3P) (acceptor) from

[1] These authors contributed equally to this work.
[2] Current address: Department of Biology, University of Isfahan, Isfahan 81746-73441, Iran.
[3] Current address: Fruit Biology and Pathology Unit, INRA-Bordeaux and Bordeaux University, 71 avenue Edouard Bourlaux, F-33883 Villenave d'Ornon, France.
[4] Current address: Plant Systems Biology Laboratory, Department of Botany and Plant Science, National University of Ireland, University Road, Galway, Ireland.
[5] Current address: J. Craig Venter Institute, Department of Microbial and Environmental Genomics, 4120 Capricorn Lane, La Jolla, CA 92037.
[6] Address correspondence to rainc@essex.ac.uk.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Christine A. Raines (rainc@essex.ac.uk).
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sedoheptulose 7-phosphate, generating xylulose 5-phosphate (Xu5P) and ribose 5-phosphate; or fructose 6-phosphate to produce Xu5P and erythrose 4-phosphate. These reactions are not only essential for the regeneration of ribulose 1,5-bisphosphate in the C3 cycle to maintain active photosynthesis, but also provide the immediate precursor molecules for the shikimic acid pathway and phenylpropanoid metabolism (erythrose 4-phosphate) and may also influence the level of G3P and ribose 5-phosphate, substrates for thiamine biosynthesis. One hypothesis that this work has raised is that increasing TK activity in the plant may have the potential to increase carbon assimilation through the C3 cycle and also to increase phenylpropanoid metabolism.

Unlike a number of enzymes of the C3 cycle, TK catalyzes a reversible reaction (Bassham and Krause, 1969; Mettler et al., 2014) and the activity of this enzyme is not known to be modulated by oxido-reduction (Stitt et al., 2010; Mittler et al., 2011). However, TK activity requires the presence of the cofactor thiamine pyrophosphate (TPP), which is synthesized from, and is the active form of the vitamin, thiamine. Biosynthesis of thiamine (and TPP) is dependent on intermediates of the C3 cycle, G3P and R5-P, both of which are substrates/products of TK. One moiety of the thiamine molecule hydroxyethylthiazole phosphate (HETP) originates from the molecule deoxyxylulose 5-phosphate (DXP), which is synthesized from G3P (from the C3 cycle) and pyruvate by the action of the first committed enzyme in the MEP pathway, deoyxylulose-5-phosphate synthase (DXS). Interestingly, the activity of DXS is itself dependent on TPP. Biosynthesis of thiamine is tightly regulated through a riboswitch on the *ThiC* mRNA (Wachter et al., 2007), which encodes an enzyme in the thiamine biosynthetic pathway. When TPP levels are high, it binds to the noncoding 3′ end of the *ThiC* mRNA, resulting in a reduction in translation thereby limiting ThiC enzyme activity. Recent studies have reported that stress leads to an increase in expression of genes for TPP biosynthesis and for enzymes that contain TPP, including TK, as well as to increased thiamine and TPP levels, suggesting a regulatory circuit exists to coordinate TK activity with availability of TPP (Rapala-Kozik et al., 2012).

The importance of the activities of individual enzymes in the C3 cycle to the distribution of carbon to central metabolic pathways is not well understood. To test this, we have generated transgenic plants with increased levels of TK activity. Study of these plants has revealed that increasing plastid TK activity causes chlorosis and negatively affects plant growth due to thiamine deficiency in the seeds of the transgenic plants.

RESULTS

Production of Transgenic Tobacco Overexpressing Plastid Transketolase

A database search identified two genes encoding plastid TK in Arabidopsis, TKL1 and TKL2. The microarray data available in the database (https://genevestigator.com/gv/plant.jsp) revealed different anatomical and developmental expression patterns for each of the Arabidopsis TKL genes. TKL1 is preferentially expressed in most organs, including photosynthetic tissues, except for senescing leaves and seeds, where *TK2* was more highly expressed (Supplemental Figure 1A). Based on these data, a full-length At-TKL1 cDNA was used to prepare a sense gene construct in the binary vector pRoK2 containing the cauliflower mosaic virus 35S promoter and NOS terminator. The recombinant vector was transferred to *Agrobacterium tumefaciens*, and this was used to transform wild-type tobacco. Primary transformant (T0) lines were selected on kanamycin-containing medium and subsequently transferred to soil and grown to maturity. Expression of the At-*TKL1* mRNA in the kanamycin-resistant plants was confirmed by RT-PCR. An increase in TK protein amounts was subsequently confirmed by immunoblot analyses (Supplemental Figure 1B). Based on these screens in the T0 generation, three TKL1-overexpressing (TKox) lines (-1, -4, and -8) were selected for further analysis and propagated by selfing through to the T3 generation.

Increased Transketolase Activity Causes Reduced Growth and Resulted in Leaf Chlorosis

In the T3 generation, all three TKox lines (-1, -4, and -8) developed a slow-growth phenotype (Figure 1A; Supplemental Figure 2) and a chlorotic phenotype (Figures 1B and 1C; Supplemental Figure 2A). The chlorosis showed two patterns of development: one in which the chlorosis extended throughout the mesophyll regions of expanding true leaves (Figure 1C; Supplemental Figure 2A) and the other with chlorosis that emerged in areas at the leaf edges (Figure 1B; Supplemental Figure 2A). The extent and location of chlorosis

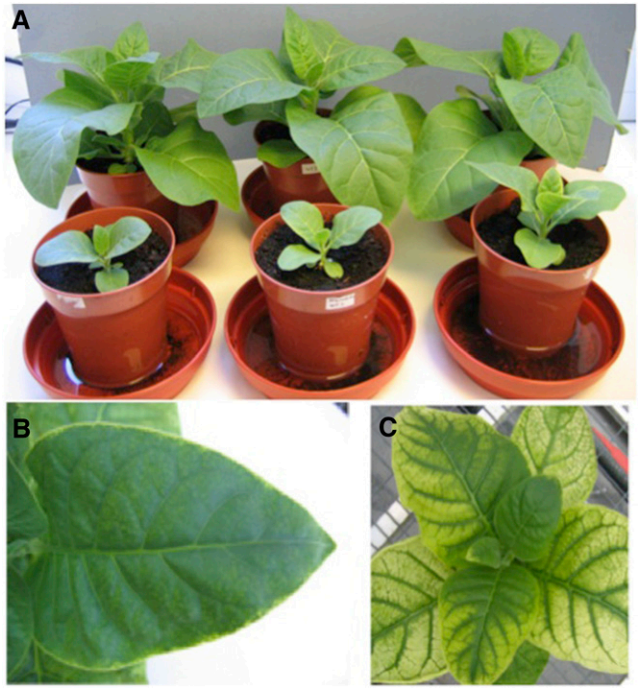

Figure 1. Phenotype of TKox Tobacco Plants.

Transgenic TKox and wild-type plants were grown in greenhouse conditions under light levels of between 600 and 1500 μmol m^{-2} s^{-1} at 25°C.

(A) Five-week-old wild-type (back row) and TKox plants (left to right TKox lines 1, 8, and 4).

(B) and **(C)** Plants at 6 to 7 weeks old show chlorosis at leaf margins (TKox4) **(B)** and in the mesophyll of mature plants (TKox1) **(C)**.

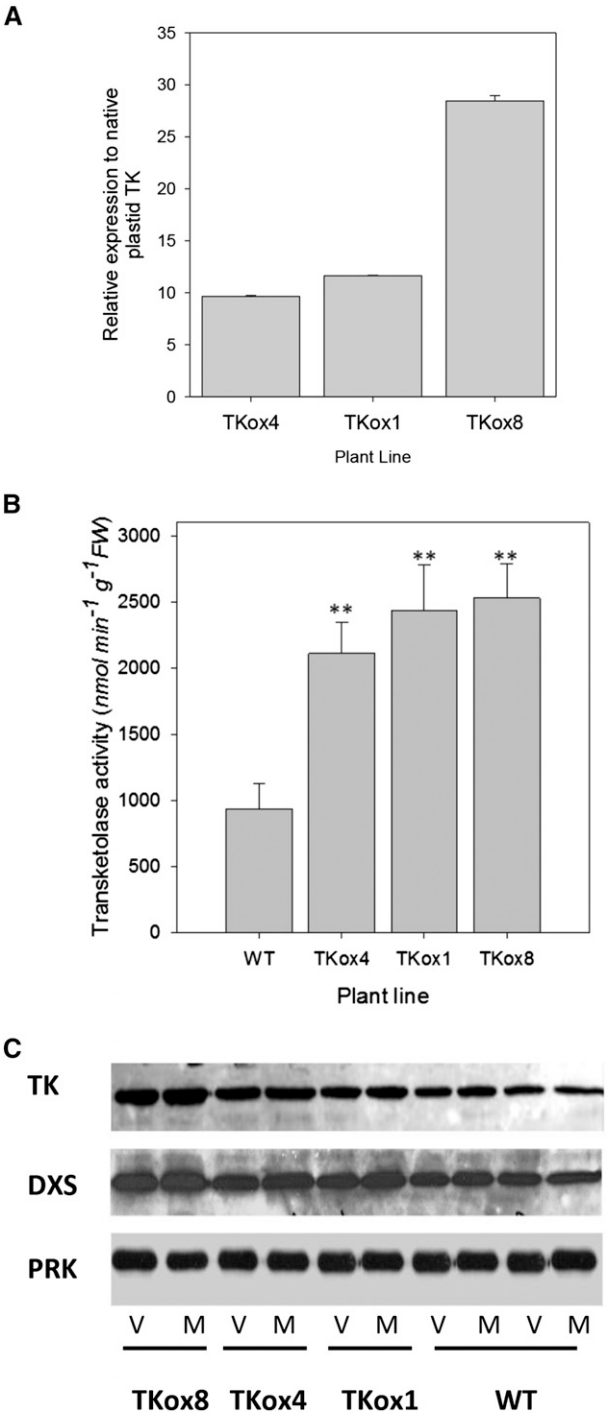

Figure 2. Molecular and Biochemical Characterization of the TKox Plants.

(A) Quantitative RT-PCR of the introduced plastid TK transgene.

(B) Total extractable TK activity in TKox and wild-type tobacco plants. The values in **(A)** and **(B)** represent the mean of four individual plants (SE ± 4) (*P > 0.05; **P < 0.01).

(C) Immunoblot analysis of protein extracts from the newest fully expanded leaf of TKox and wild-type plants. M, mesophyll; V, vein tissue extracts. Proteins (5 μg) were separated on a 12% polyacrylamide gel and blotted onto nylon membrane and probed with polyclonal antibodies against TK, DXS, and PRK proteins.

varied between siblings from a given transformation event. In young plants, the chlorotic phenotype was less severe but growth of roots and shoots were retarded (Supplemental Figure 2B).

Quantitative RT-PCR confirmed high levels of expression of the introduced transgene in the lines TKox-1, -4, and -8, with 10- to 30-fold more transcripts for At-TKL1 than the endogenous tobacco Nt-*TK* (Figure 2A). Immunoblot analysis revealed increased levels of TK protein in these lines (Supplemental Figure 3). TK enzyme assays confirmed that these plants had increased TK activity compared with that of wild-type plants, although the differences (1.76- to 2.5-fold increase compared with wild-type plants) in TK activity were less pronounced than the differences in transcript abundance (Figure 2B; Supplemental Table 1). TK activity was assayed in optimal conditions, and it has been shown that such measurements provide a proxy for protein abundance (Piques et al., 2009). Immunoblot analysis confirmed that TK protein levels were increased in both the green and chlorotic sectors of leaves of all transgenic lines compared with that of wild-type plants (Figure 2C). While not quantitative, the increase is of the same order as that indicated by activity measurements. Immunoblotting showed that the TKox lines had increased levels of DXS protein, but did not show any significant change in protein abundance for the Calvin cycle enzyme PRK (Figure 2C). This analysis also showed that levels of TK protein are increased to a similar extent in both the green and yellow regions (Figure 2C). This shows that the pattern of chlorosis evident in the TKox plants was not due to differential expression of the sense construct or to cosuppression.

Photosynthetic Carbon Assimilation Rates and Carbohydrate Levels Are Similar to the Wild Type

The chlorotic phenotype in the TKox lines at the vegetative stage (Figure 1) made it difficult to assess the direct effect of increased TK activity on photosynthetic capacity in mature plants. To avoid this, we determined the light-saturated CO_2 assimilation rates under a range of CO_2 concentrations using 4- to 5-week-old TKox-1, -4, and -8 plants that displayed only mild chlorosis (Supplemental Figure 2A). The data show that a typical relationship between C_i concentration and CO_2 is observed in both the wild-type and TKox lines and that the assimilation rate in TKox plants is similar to that of wild-type plants (Figure 3).

The rate of photosynthesis in 4- to 5-week-old TKox plants was similar to that of the wild type; thus, to explore further the cause of the slow-growth phenotype, the effect on the levels of starch and soluble sugars was investigated. In a preliminary study, TKox and wild-type plants were grown in either short (8 h light/16 h dark) or long (16 h light/8 h dark) days. Leaves were sampled from 4-week-old plants and subjected to iodine staining. Whereas starch remained at the end of the night in TKox plants in both long- and short-day conditions, no starch (short-day growth) or lower levels (long days) were detectable at the end of the night in the wild type (Supplemental Figure 4). To investigate if the increase in starch in the TKox plants was due to changes in rates of accumulation or degradation, we set up a second experiment. Plants were grown in

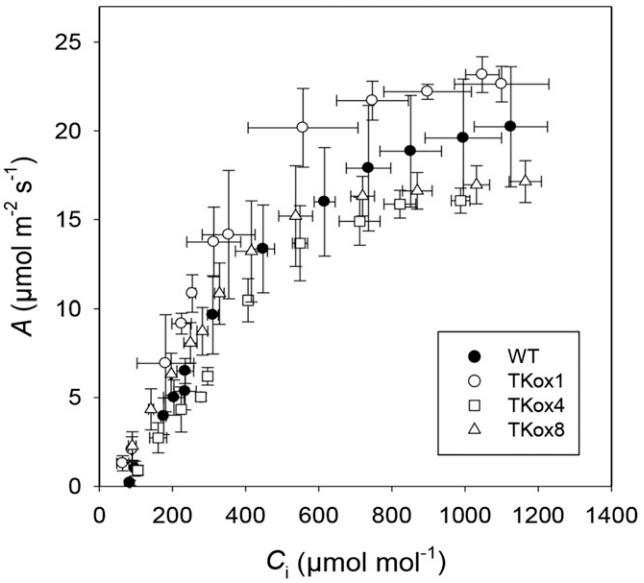

Figure 3. Photosynthetic Rate as a Function of Internal CO_2 Concentration in TKox and Wild-Type Tobacco Plants.

Plants were grown in controlled environment conditions for 5 weeks (16 h light/8 h dark) with light levels of 200 to 250 μmol m^{-2} s^{-1}. Photosynthetic carbon fixation rates were determined in the newest fully expanded leaves as a function of increasing CO_2 concentration at saturating light levels. Values represent the mean of at least three plants (±SE).

long days (16-h photoperiod) for 5 weeks, placed in the dark for 24 h (to allow starch levels to decline), returned to a light-dark cycle, and leaves sampled at six time points over the following diurnal cycle. Although the general pattern of accumulation of starch over the diurnal cycle was similar in the wild-type and TKox plants, the TKox lines 1 and 8 had higher levels of starch than wild-type plants at 12:00 AM and 6:00 AM ($P < 0.05$; Supplemental Table 2; Figure 4). No significant difference was observed in either the pattern of accumulation or the maximum amount of sucrose accumulated at the end of the light period between TKox and wild-type plants (Supplemental Table 2; Figure 4). While the pattern of accumulation of glucose and fructose was similar in the TKox lines compared with wild type, all three TKox lines accumulated between 30 and 50% more glucose and fructose than the wild-type plants ($P < 0.05$; Supplemental Table 2; Figure 4). Analysis of growth of the TKox lines at four developmental stages showed that throughout development, the TKox plants had reduced leaf area, root length, and total dry weight when compared with wild-type plants grown in the same conditions (Figure 5).

Shikimic Acid Pathway Intermediates Aromatic Amino Acids and Phenylpropanoids

In a previous study in which the level of TK activity was reduced by antisense technology, a dramatic decrease in the levels of intermediates in the phenylpropanoid pathway was observed. To assess if the slow growth and chlorotic phenotype was related to changes in this pathway, a range of intermediates were monitored. This revealed no major changes in the levels of amino acids, including aromatic amino acids, and phenylpropanoids between the TKox and wild-type plants (Supplemental Table 1).

Intermediates of the C3 Cycle Do Not Rescue the TKox Phenotype

To explore the basis of the chlorotic and growth phenotype further, TKox plants were germinated and grown in a range of environmental conditions, including different daylengths and light intensities (data not shown; see Methods for details). None of the conditions in which the plants were grown prevented development of the chlorotic or slow-growth phenotype. In addition, TKox plants were supplied with intermediates of the C3 cycle: Xu5P and G3P and also with pyruvate. None of these metabolites were able to rescue the chlorotic cotyledon phenotype (data not shown; see Methods for details). Interestingly, DXP supplementation (the first committed intermediate in the isoprenoid pathway) partially rescued the phenotype, but only when plants were provided with this metabolite continuously from sowing (Figure 6).

The Chlorotic and Slow-Growth Phenotypes Are Rescued by Supplementing TKox Plants with Thiamine or TPP

The cofactor TPP is essential for TK activity. TPP is the active form of thiamine and is synthesized from precursors that are provided by activity of TK in the C3 cycle or the oxidative pentose phosphate pathway. One hypothesis for the chlorotic and slow-growth phenotypes of TKox plants would be that increased activity of TK disrupts carbon allocation and the availability of the precursors for biosynthesis of TPP. To test this, TKox lines 1 and 8 and wild-type plants were grown on Murashige and Skoog (MS) media with or without TPP (10 mg L^{-1}). The results clearly showed that the chlorotic phenotype evident in the cotyledons was alleviated and the growth improved, when the TKox plants were germinated in the presence of TPP (Figure 7; Supplemental Figure 5A).

Photosynthetic capacity was compared in the cotyledons and the first true leaves of TKox and wild-type seedlings (10 d after germination), grown in media with or without TPP, using chlorophyll fluorescence imaging. The data showed that the maximum quantum efficiency of photosystem II (PSII) photochemistry, F_v/F_m, and the operating efficiency of PSII photochemistry, F_q'/F_m', were significantly reduced in TKox lines grown in normal media, compared with the equivalent wild-type plants (Figure 7B; Supplemental Figures 5A and 5B). Whereas the fluorescence signals were relatively homogeneous in wild-type plants, the TKox lines showed variable fluorescence signals. By contrast, TKox plants grown in media supplemented with TPP (10 mg L^{-1}) had a similar F_v/F_m and F_q'/F_m' to that of wild-type plants. The inhibition of photosynthesis in TKox in this experiment and the absence of an inhibition of photosynthesis in the experiment of Figure 4 can be explained because in the current experiment the plants exhibited chlorosis, whereas for the initial studies in Figure 4, we used plants with a very mild chlorosis phenotype.

In addition to chlorosis and decreased shoot growth, the TKox lines also showed a large decrease in root growth (Supplemental Figure 6). This phenotype was also fully rescued by thiamine supplementation (Supplemental Figure 6). Taken together, these data suggest that the TKox plants are partial thiamine auxotrophs.

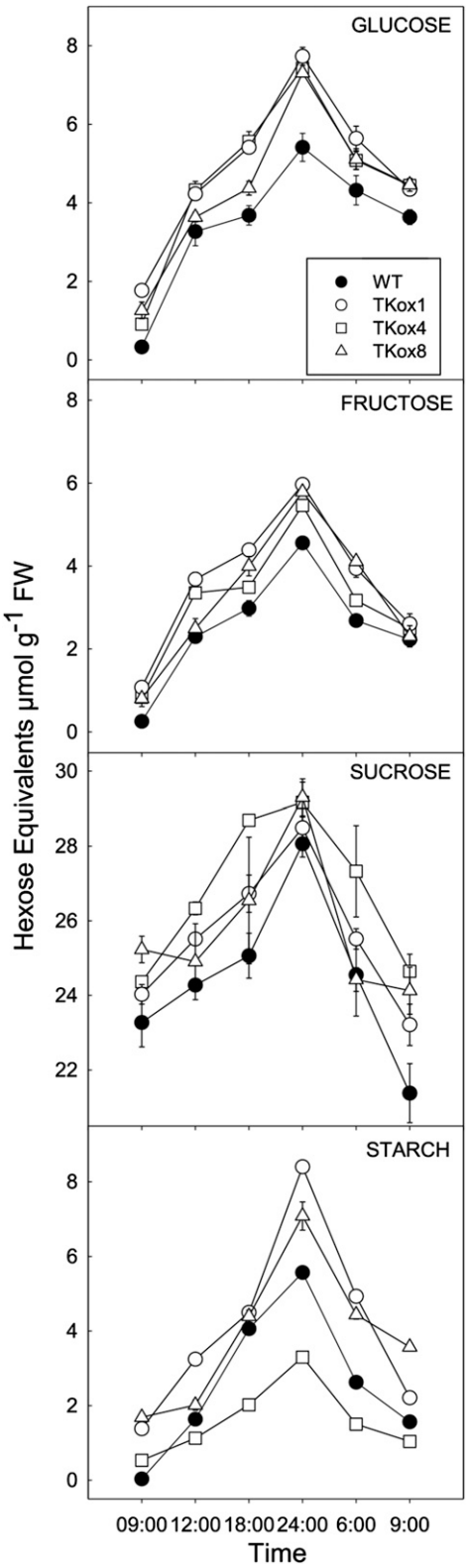

Figure 4. Diurnal Carbohydrate Accumulation in TKox and Wild-Type Tobacco Plants.

Plants were from the same set as those used in Figure 3. Whole plants were harvested at six time points over a diurnal cycle and levels of glucose, sucrose, and starch determined. Each value represents the mean of four measurements ± SE.

The Effect of TPP and Thiamine on the Phenotype of TKox Plants Is Dependent on the Timing of Supplementation

We next tested whether thiamine supplementation was necessary only during germination and the early stages of growth or if it was required throughout the entire life cycle. TKox lines 1 and 8 and wild-type seedlings were germinated on supplemented or unsupplemented media for 12 d and then transferred onto soil. Provision of thiamine or TPP during germination and early growth complemented both the chlorotic and the growth phenotype, and this was maintained up to the 6th leaf stage 30 d after germination (Supplemental Figure 7). At later stages, the chlorotic and growth phenotype returned (Supplemental Figure 7B).

In a second experiment, we repeated this analysis using all three TKox lines and wild-type plants and also asked if continued provision of thiamine prevented the return of the chlorosis and growth phenotype. Plants were germinated and grown in MS medium with or without thiamine supplementation for a period of 12 d, and following this, seedlings were transferred to soil and irrigated with either water alone or water with thiamine (Figure 8). TKox lines grown in the absence of thiamine during germination and after transfer to soil (−/−) displayed a chlorotic phenotype and were smaller than wild-type plants (Figure 8). Interestingly, plants that were germinated without thiamine but were supplemented with thiamine following transfer to soil (−/+) produced small plants similar to or slightly larger in size to the −/− plants but in this case the chlorotic leaf phenotype was complemented (Figure 8). TKox plants germinated in the presence of thiamine and then transferred to soil and given only water (+/−) grew to a size similar to that of the plants in which the supplementation regime had been continued in soil (+/+), but as they grew, a chlorotic leaf phenotype became apparent (Figure 8).

We quantified the effect of TPP supplementation on growth of the TKox and wild-type plants by measuring the shoot diameter as a proxy for biomass. In this experiment, we focused on the two lines with the most severe phenotype. This experiment showed clearly the importance of timing of supplementation with TPP in the early period for growth for rosette size. TKox lines supplemented with thiamine up to day 12 had similar shoot diameter to the wild type, irrespective of whether the supplementation was continued after 12 d or not, and TKox lines that were not supplemented with thiamine in the first 12 d were smaller than wild-type plants, even if they were supplemented with thiamine form 12 d onwards (Supplemental Figure 8). At 34 d after germination, the plants were destructively harvested and leaf areas and dry weights determined. This analysis showed that in +/+ and +/− conditions, TKox shoot weight and leaf area was either similar to or greater than that of the wild type, whereas in both −/+ and −/−, shoot weight and leaf area were much smaller in TKox than in the wild type (Supplemental Figure 9).

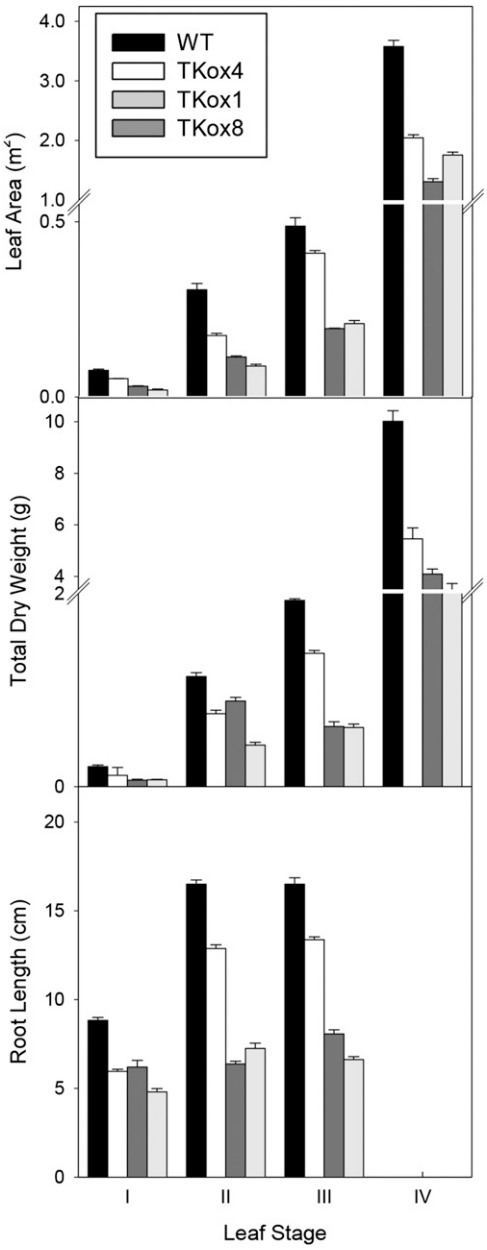

Figure 5. Growth Analyses of the Wild-Type and TKox Tobacco Plants Grown in Controlled Environment Conditions.

Plants were grown in the same conditions as Figure 3. Analyses were performed at four developmental stages: 3 (I), 5 (II), 7 (III), and 11 leaves (IV). Each value represents the mean of four individual measurements ± SE. Data are not available for stage IV roots.

Thiamine Levels in Seeds and Cotyledons of Germinated Seedlings Are Reduced in the TKox Plants

Analyses of thiamine in the seeds of the wild type and TKox lines 1 and 8 revealed that the levels of thiamine in the TKox plants was less than 50% of that in wild-type tobacco seeds (Figure 9). TPP was below detection in the seeds of both wild-type and TKox lines (data not shown). Imbibition of TKox seeds in the presence of thiamine, followed by a series of washes, restored the levels of thiamine found in seed extracts to that of wild-type seeds. This treatment of the seeds also rescued both the chlorotic and growth phenotype of the developing TKox line 1 seedlings (Supplemental Figure 10). We also investigated if the low-thiamine seed phenotype could be rescued by thiamine supplementation of growing plants. TKox lines 1 and 8 +/+ plants produced seeds containing thiamine levels equivalent to those of wild-type plants (Figure 9).

We also analyzed thiamine and TPP levels after germination in cotyledons at stages ST.1 (cotyledons 3 mm at widest point and no first true leaf evident) and ST.2 (cotyledon fully expanded and 1st true leaf visible) (Figure 10). The level of thiamine in all three TKox lines was significantly below that of wild-type plants at both stages of cotyledon development. At Stage 1 (ST1), thiamine levels in TK4 plants were 40% that of the wild type, while TKox lines 1 and 8 had <20% of wild-type thiamine content. Comparing TPP levels in the TKox lines with that of the wild type revealed a different pattern. At developmental ST1, the levels of TPP in TK4 were equivalent to that of the wild type, and TPP contents in TKox lines 1 and 8 were significantly lower, ~50 to 60% that of the wild type. In fully expanded cotyledons (stage 2), TPP levels in the three TKox lines showed only a small (20%) and nonsignificant decrease

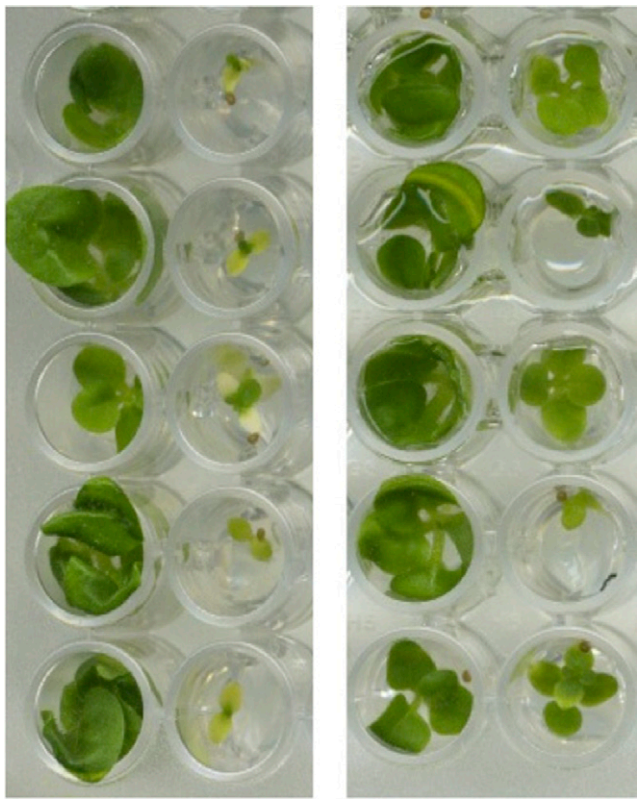

Figure 6. DXP Supplementation Rescues the Chlorotic Phenotype of TKox Plants.

Seedlings were germinated in MS media with (right) and without (left) additional DXP (2 mM), placed in a controlled environment chamber, and the growth of the seedlings recorded 13 d after germination.

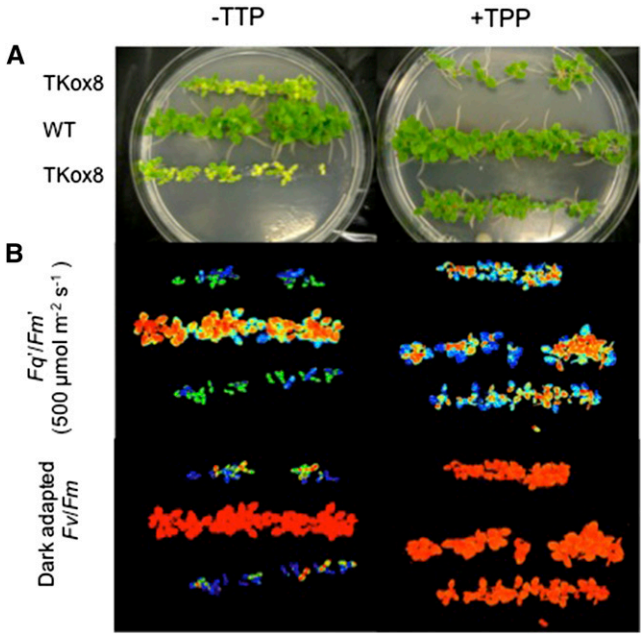

Figure 7. Complementation of the TKox Phenotype with TPP.

(A) Appearance of the TKox and wild-type plants grown in MS media for 12 d with or without TPP (10 mg L^{-1}).
(B) Chlorophyll fluorescence imaging of dark-adapted TKox and wild-type plants of a duplicate set of plants grown as in **(A)**.

compared with the wild type (Figure 10). On average, the seedlings required between 6 and 11 d in total to reach stage 1 and stage 2, respectively.

Gene Expression Changes in TKox Plants

The 3′ untranslated region (UTR) of *ThiC*, encoding the enzyme thiamine synthase C, contains a TPP-regulated riboswitch. When TPP levels are high, TPP binds to the 3′ UTR and alternative splicing of the pre-mRNA encoding the ThiC protein occurs. This results in the production of three different transcripts: the coding sequence (*ThiC1*), intron retention variant (*ThiC2*) (increases when TPP is low), and an intron splice variant (*ThiC3*) (decreases when TPP is low) (Wachter et al., 2007). The tissue used in the experiments in Figures 11 and 12 was taken from seedlings within the ST1 development stage as changes in TPP levels were most pronounced at this point. In all three TKox lines, the abundance of the *ThiC2* splice variant was higher than wild-type plants, while that of *ThiC3* transcript was decreased substantially and consistently in cotyledons from both 3- and 9-d-old seedlings. Taken together, these results suggest that the pattern of expression of the *ThiC* alternative transcripts in the TKox lines is consistent with a low TPP phenotype (Figure 11).

We also asked if overexpression of At-*TK* affected the levels of the endogenous Nt-*TK* or transcripts of the other TPP-regulated enzymes. As with most of the transcripts studied, no significant change in the level of Nt-*TK* transcripts relative to that in wild-type plants was observed in the TKox plants at either 3 or 9 d after germination (Figure 12). However, in TKox plants, the *DXS* transcript levels were approximately double that seen in the wild type at the 3-d-old stage but were half that of the wild type in 9-d-old cotyledons. *PDC* levels in TKox plants were lower than the wild type after growth for 3 d, but equivalent to the wild type at the 9-d-old stage. Conversely, *BCKDC* transcript levels were equivalent to that of the wild type at 3 d, but lower at 9 d (Figure 12).

DISCUSSION

Increased TK Activity Has a Small Negative Effect on Photosynthetic Carbon Assimilation but Dramatically Reduces Shoot and Root Growth

In this study, we have shown that increasing the expression of Arabidopsis plastid TK in transgenic tobacco has unexpected results. The TKox lines had a high level of transcript and protein for the introduced Arabidopsis TK, unaltered levels of transcript for the equivalent endogenous tobacco *TK* gene, and a 1.76- to 2.5-fold increase in total TK activity assays in optimized conditions in leaf extracts. However, the TKox plants developed a chlorotic phenotype in the mesophyll cells and leaf margins and were smaller than wild-type plants. These results are in sharp contrast to the expected gain in photosynthetic rate, which we hypothesized based on an earlier TK antisense study in which a small decrease in TK activity led to an inhibition of

Figure 8. Timing of Thiamine Supplementation Is Critical to Rescue the TKox Phenotype.

TKox and wild-type plants were germinated in media containing thiamine (50 mg L^{-1}) and then transferred to soil 12 d after germination and watered with thiamine (+/+) or without (+/−). A second set of plants were germinated without thiamine and watered with thiamine (−/+) or without (−/−). Plants were grown in greenhouse conditions under light levels of between 600 and 1500 µmol m^{-2} s^{-1}, 25°C. Photograph was taken after 34 d in soil.

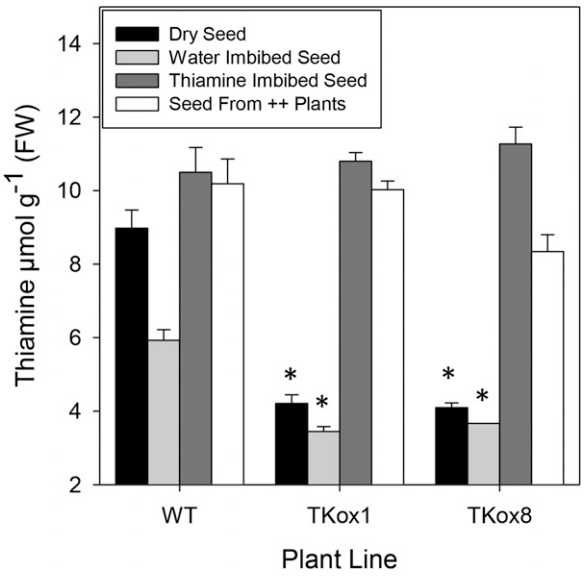

Figure 9. Thiamine Levels Determined in TKox and Wild-Type Seeds.

Seeds were analyzed for thiamine content when dry or following 24 h in thiamine (50 mg L^{-1}). The parental +/+ plants were watered with thiamine (50 mg L^{-1}) throughout the life cycle. Statistical comparisons to wild-type values were made using a one-way ANOVA (95% sig) and Tukey post hoc test. Asterisk indicates significance at $P < 0.05$. Results are the mean ± SE ($n = 4$).

photosynthesis and studies showing that overexpression of the C3 cycle enzyme SBPase results in an increase in photosynthetic activity and biomass (Henkes et al., 2001; Lefebvre et al., 2005; Raines, 2011).

Photosynthetic carbon assimilation rates, measured under growth conditions in the young TKox transgenic plants, were not significantly different to wild-type plants, provided that care was taken to avoid inclusion of chlorotic leaf regions to measure photosynthesis rates. However, in the seedlings where strong chlorosis was evident, the overall rate of photosynthesis was decreased. The levels of starch accumulation over the diurnal cycle were higher than in wild-type plants in TKox lines 1 and 8, while sucrose and reducing sugars either accumulated more rapidly in the first part of the light period or were higher at the end of the light period. Although leaf chlorosis has previously been seen in transgenic tobacco lines that accumulate a large amount of soluble carbohydrates in the leaves (von Schaewen et al., 1990; Riesmeier et al., 1993; Lerchl et al., 1995), the levels of starch and soluble sugars in the TKox leaves were far lower than in these transgenic lines, making it likely that other factors are responsible for the chlorosis in the TKox lines.

Analysis of antisense *TK* plants revealed that carbon allocation from the C3 cycle to the shikimate pathway was decreased linearly with decreased TK activity (Henkes et al., 2001). One hypothesis to explain the unexpected decrease in growth and development of chlorosis in the TKox lines would be that increasing TK activity may preferentially divert carbon flow toward the shikimate pathway, thus causing a restriction of intermediates for the other pathways and subsequently a detrimental effect on growth. However, analysis of phenylpropanoid and amino acid levels in the TKox plants did not reveal any major changes in the levels of intermediates or end products of the shikimate pathway, such as aromatic amino acids or several major phenylpropanoids. The implications of these findings are that the chlorosis and slow growth in TK-overexpressing lines are not the result of reduced photosynthetic capacity nor are they due to accumulation of carbohydrates or to changes in flux through the shikimic acid pathway; therefore, the cause must lie elsewhere.

TKox Plants Display Partial Thiamine Auxotrophy

Here, we show that the striking chlorotic and growth phenotype of the TKox plants can be complemented by addition of thiamine or TPP to the growth medium, providing evidence that increased plastid TK expression resulted in a thiamine auxotrophic phenotype. This finding is in agreement with studies of Arabidopsis thiamine mutants, which showed that the chlorotic appearance could be alleviated when the plants were fed with thiamine (Li and Rédei, 1969). Tobacco mutants in thiamine biosynthesis exhibited a 50% reduction in chlorophyll pigments that was also fully reversible by the addition of exogenous thiamine (McHale et al., 1988). A similar result was also obtained in the Arabidopsis *ThiC* insertion mutants that were blocked in the biosynthesis of the pyrimidine moiety of the thiamine molecule, producing albino plants that exhibited slow growth, even though starch and sucrose levels accumulated up to 40 and 50% more, respectively, than wild-type plants (Raschke et al., 2007; Kong et al., 2008).

We also provide evidence showing that one major reason for the growth phenotype of the TKox lines is reduced availability of thiamine in the seed. This was demonstrated by the finding that imbibition of seeds in the presence of thiamine, with subsequent germination and growth on nonsupplemented media, was sufficient to complement the growth phenotype of the TKox plants. Provision of thiamine or TPP at the early stage of germination could also almost completely complement this phenotype. However, if thiamine or TPP were not provided until 12 d after germination, the growth phenotype was never completely restored to that of the wild type. This shows clearly that a process required for this early growth was irreversibly affected by the reduced availability of thiamine during early postgerminative growth. Thiamine stored in seeds is essential for early biosynthesis of TPP for the activation of a range of metabolic enzymes needed for the remobilization of stored reserves in seeds to power germination, seedling establishment, and early plant development. TPP acts as a cofactor for a number of enzymes in central cellular metabolic pathways, including glycolysis, the pentose phosphate pathway, the citric acid cycle, and the C3 cycle, in addition to isoprenoid biosynthesis in the MEP pathway.

Following on from this is that the inability of thiamine to rescue the TKox slow-growth phenotype of TKox plants when they are older than 12 d after germination may be due to impairment of development through a detrimental effect on metabolism in the meristem during early postgermination growth (Woodward et al., 2010). Evidence to support this proposal comes from the analysis of a maize (*Zea mays*) thiamine mutant that showed that

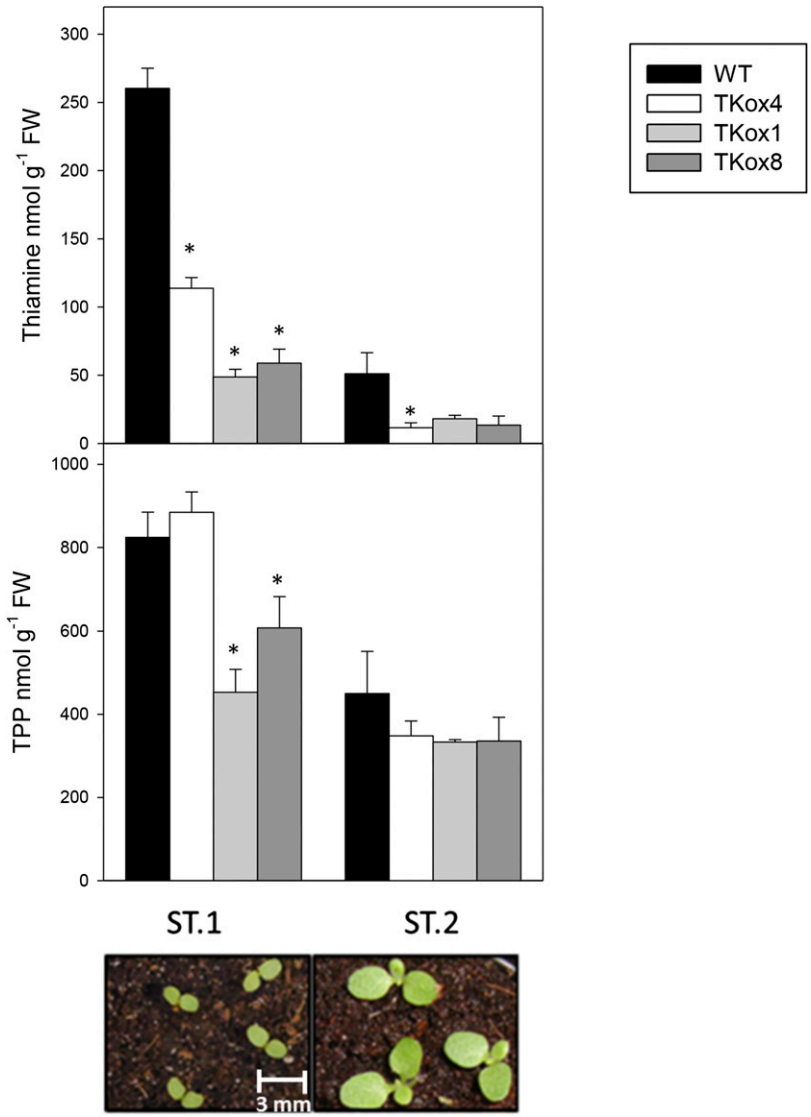

Figure 10. Thiamine and TPP in TKox and Wild-Type Cotyledons.

Seeds were germinated on compost and plants were grown in a controlled environment chamber at 25°C and light levels of 300 m^{-2} s^{-1} with a 12-h photoperiod. Samples were taken midpoint of the photoperiod. Thiamine/TPP content was determined in leaves at two time points following germination: ST.1 (cotyledons 3 mm at widest point across both and no first true leaf evident) and ST.2 (cotyledon fully expanded and 1st true leaf visible). Statistical comparison to the wild type was made using a one-way ANOVA (95% sig) and Tukey post hoc test. Asterisk indicates significance at $P < 0.05$. Results are the mean ± SE ($n = 4$).

development and maintenance of the shoot apical meristem is dependent on thiamine biosynthesis within developing leaves, which are needed to provide the TPP for metabolism in the meristematic cells (Woodward et al., 2010). In mammalian systems, it has been observed that TK activity is an important determinant of cell proliferation in cancers (Deberardinis et al., 2008). However, a role for TK in plant meristem function or growth has not yet been identified. One important finding from the work presented here is that thiamine availability during germination and early seedling establishment is not only essential but cannot be fully compensated for at later stages of development, pointing to an irreversible impairment of one or more key processes that are required for plant growth.

TKox Plants Display Compromised Carbon Allocation to the Isoprenoid and Starch Biosynthetic Pathways

Our results showed that TKox plants exhibit significant changes in the amount of chlorophyll, thiamine, and TPP, have elevated levels of soluble sugars, and show a slight starch-excess phenotype (Supplemental Figure 4). The chlorosis phenotype of the TKox lines could also be rescued by thiamine addition. Interestingly,

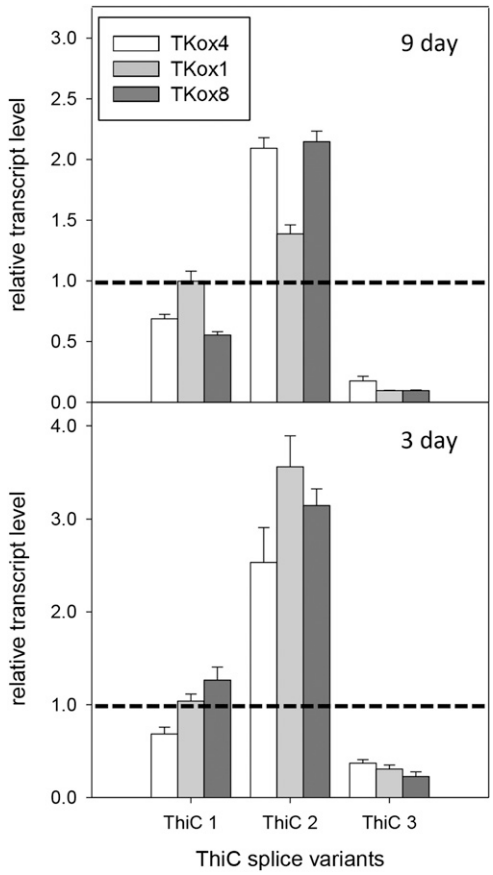

Figure 11. qPCR Analysis of Transcript Levels of *ThiC*.

Relative levels of the splice variant transcripts of the *ThiC* gene (*ThiC1*, *ThiC2*, and *ThiC3*) were determined by qPCR; the dashed line indicates the wild type. The values represent a pooled sample of whole plant material taken at 3 and 9 d after germination. The results are the mean from three technical replicates and the error bars indicate the SE.

this rescue was also possible when thiamine was not supplied until later than 12 d after germination, indicating that it may involve a different requirement for thiamine than the defect that affects growth. These results also show that the decrease in growth is not primarily due to chlorosis because growth was still inhibited even when thiamine addition from 12 d onwards rescued the chlorotic phenotype. This is consistent with our proposal (see above) that the slow growth is a consequence of the low thiamine.

Chlorophyll and carotenoids are biosynthesized in the MEP pathway, and the first reaction of this pathway is catalyzed by DXS, which combines G3P from the C3 cycle with pyruvate from the glycolytic pathway to form DXP. G3P is the substrate for the TK reaction in the C3 cycle and is the product of the TK reaction in the oxidative pentose phosphate pathway, placing this enzyme at a crucial interface between primary carbon metabolism and the pathways it feeds. Furthermore, not only is DXS activity dependent on the presence of TPP, but the product of the DXS reaction is DXP, which is the first substrate in the biosynthesis of the HETP moiety of the thiamine molecule and hence TPP (for review, see Julliard and Douce, 1991; Julliard, 1992) (Figure 13). One explanation for the chlorotic phenotype in the TKox plants could be that carbon flow to the MEP pathway is restricted due to the increased activity of TK, which negatively affects the substrates available for TPP biosynthesis. Evidence to support this comes from our feeding experiment that revealed that provision of DXP in the growth media of TKox seedlings with fully expanded cotyledons complemented the chlorotic phenotype. However, unlike provision of thiamine, the chlorosis returned as the true leaves developed, suggesting that the continuous supply of the substrate was needed to sustain chlorophyll and thiamine biosynthesis.

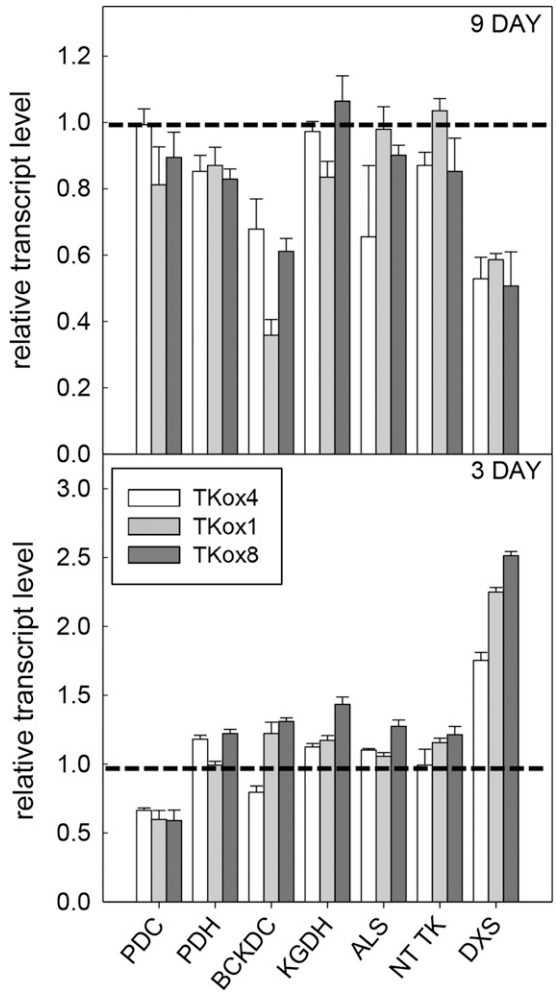

Figure 12. qPCR Analysis of Transcript Levels of Thiamine-Dependent Enzymes.

The values represent cDNA comprising equal quantities of RNA from three independent samples of pooled plant material from each line (the wild type, TKox-4, -1, and -8). PDC, pyruvate decarboxylase; PDH, pyruvate dehydrogenase; BCKDC, branched-chain α-ketoacid dehydrogenase; α-KGDH, α-ketoglutarate dehydrogenase; ALS, acetolactate synthase; NT-TK, native plastid transketolase. The values represent a pooled sample of whole-plant material taken at 3 and 9 d after germination. The results are the mean from three technical replicates, and the error bars indicate the SE.

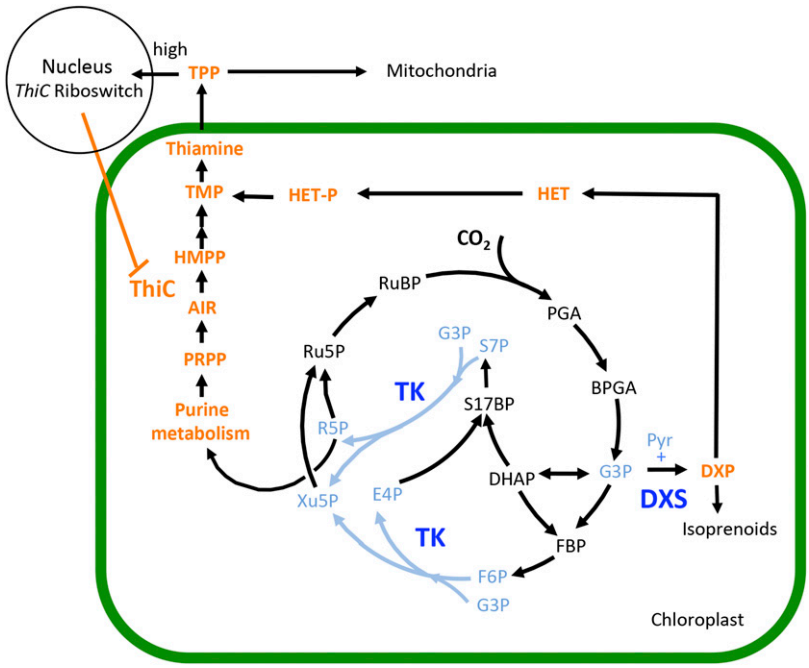

Figure 13. The C3 Cycle Showing Relationships to the Isoprenoid and Thiamine Pathways.

Thiamine biosynthesis (red typeface) is formed from two moieties, HMPP and HETP, that are biosynthesized from compounds arising from the C3 cycle, ribose 5-phosphate (R5P), and G3P. These two compounds are a product and substrate, respectively, of the reactions performed by TK. Thiamine monophosphate is generated from the condensation of HETP and HMPP. Thiamine monophosphate is dephosphorylated to thiamine, which is transported to the cytosol where it undergoes phosphorylation to TPP. TPP can bind to the riboswitch regulatory mechanism of *ThiC* located in the 3′ UTR of nascent mRNA in the nucleus, resulting in a reduction of biosynthesis of the ThiC protein and in turn the biosynthesis of thiamine. TPP-requiring enzymes are shown in bold in dark blue.

The starch level in the TKox plants was increased, both at dawn in short days when starch is effectively exhausted in wild-type plants (Supplemental Figure 4) and when plants were re-illuminated after a period of darkness (Figure 4D). This could simply be a consequence of the slow-growth phenotype of these plants. However, starch and chlorotic phenotypes have been observed in plants with altered thiamine metabolism, even in those where growth was not reduced and that possessed an increased capacity for TPP biosynthesis (Bocobza et al., 2013). Plants expressing a *ThiC* gene with a mutated riboswitch that was insensitive to TPP had starch grains with altered structure, suggesting a change in the process of starch biosynthesis. Interestingly, plants overexpressing a wild-type *ThiC* gene displayed a similar chlorosis and starch accumulation phenotype to the mutated riboswitch lines; however, the growth of these plants appeared to be slower than the wild type. It should also be noted that the TK antisense plants appeared to maintain starch levels while sucrose dropped; this is in contrast to our normal expectation of the relationship between starch and sucrose (Goldschmidt and Huber, 1992; Haake et al., 1998; Henkes et al., 2001). Taken together, these results would suggest that changes in starch accumulation in plants with altered thiamine metabolism may not be simply due to slow growth.

An alternative explanation for the chlorotic phenotype is that overexpression of TK leads to sequestration of TPP on the TK protein, resulting in a lack of TPP for other enzymes, including DXS. The enzymes of the C3 pathway are present at high abundance, 10-fold or more above the abundance of other enzymes in primary metabolism and more so compared with pathways like the MEP pathway (Sulpice et al., 2010; Baerenfaller et al., 2012; Mettler et al., 2014). The level of thiamine and TPP measured in tobacco cotyledons was ~250 and 800 nmol g^{-1} fresh weight (FW) at ST.1, respectively, falling to 50 and 400 nmol g^{-1} FW at ST.2 (Figure 10). For comparison, the abundance of TK binding sites has been estimated as ~3.4 nmol g^{-1} FW in spinach (*Spinacia oleracea*) leaves (Harris and Königer, 1997) and 2.5 to 4.4 nmol g^{-1} FW in Arabidopsis rosettes (Piques et al., 2009). It therefore seems unlikely that overexpression of TK decreases thiamine and TPP by simple sequestration.

Conclusion

This report demonstrates that thiamine auxotrophy in plants can be caused by overexpression of a TPP-dependent enzyme. This appears to be a unique feature of a C3-cycle enzyme, as overexpression of SBPase, FBP aldolase, or a cyanobacterial bifunctional FBPase/SBPase did not have this negative effect (Miyagawa et al., 2001; Lefebvre et al., 2005; Uematsu et al., 2012). These data together with that from the *TK* antisense study have revealed that plants can tolerate only small changes in the level of the endogenous TK protein; otherwise, there is an effect the pathways that receive compounds from the C3 cycle (Henkes et al., 2001). In the case of the antisense plants, a 20 to 40% decrease leads to reduced levels of shikimate and phenylpropanoid pathway products, and

in TK-overexpressing lines an ~1.76- to 2.5-fold increase in TK leads to a reduction in thiamine and TPP levels (Henkes et al., 2001). The implication of this is that there is a mechanism that determines the levels of plastid TK protein and maintains it within a narrow range. However, as yet this mechanism is not known.

Recent studies on thiamine metabolism in photosynthetic organisms have revealed complex regulatory mechanisms involving a riboswitch located on the *ThiC* pre-mRNA and the presence of regulatory elements located in the 5′ upstream sequence of this gene responsible for circadian control of transcription (Bocobza et al., 2013). In addition, a link between stimulation of thiamine biosynthesis under stress and the induction of mRNA for TK and other TPP-requiring enzymes was recently reported (Rapala-Kozik et al., 2012). What is clear from these analyses is that thiamine biosynthesis is a highly regulated process involving light, stress, circadian rhythms, and a TPP riboswitch. Here, we show that plastid TK is also an important part of this regulation. No major changes in the levels of transcripts encoding the TPP-requiring enzymes or enzymes of the thiamine biosynthetic pathway were evident in the TKox plants, suggesting that increased TK activity alters flux through the biosynthetic pathway for thiamine. Support for this comes from our finding that provision of DXP partially compensates for the chlorotic phenotype. In addition, balancing the provision of both of the moieties hydroxymethylpyrimidine pyrophosphate (HMPP) and HETP used for biosynthesis of thiamine has recently been shown to be crucial. In *Chlamydomonas reinhardtii*, the *ThiC* riboswitch is sensitive not only to TPP but also to HMPP, and in addition, the *THI4* gene responds to thiazole (Moulin et al., 2013; Pourcel et al., 2014). Although these regulatory steps have not been shown in land plants, this work highlights the complex regulatory system to ensure that sufficient, but not excess thiamine is available. These findings also highlight the importance of the activity of the enzymes of the C3 cycle in determining plant growth and development.

Importantly, this study suggests that increase TK activity without detrimental effects (loss of biomass), it might be necessary to add TPP-responsive regulatory elements in the genetic *TK* construct and/or concomitantly overexpress enzymes of the TPP biosynthetic pathway. This is a significant finding as it highlights that engineering plants for desired traits, such as improving photosynthetic carbon assimilation or thiamine biofortification, will require a deeper understanding of the molecular components and regulatory mechanisms controlling these processes (Raines, 2011; Pourcel et al., 2014). Leveraging this knowledge is critical for the successful design and complex engineering of plants with enhanced desirable traits.

METHODS

Generation of the Transgenic Plants

An *Arabidopsis thaliana* full-length plastid transketolase cDNA TKL1 was cloned into the pMog 22 vector containing the cauliflower mosaic virus 35S promoter and nos terminator sequences. The recombinant plasmid was introduced into tobacco (*Nicotiana tabacum* cv Samsun) using *Agrobacterium tumefaciens* LBA4404 via leaf disc transformation (Horsch et al., 1985). Shoots were regenerated on selective medium containing kanamycin (100 mg L^{-1}), and primary transformants (T0) were allowed to self-fertilize. Plants were then selected and self-fertilized twice more to generate lines of the third generation (T3).

Plant Material and Growth Analysis

The seeds of *N. tabacum* cv Samsun wild-type and mutant lines (TKox lines -1, -4, and -8) were sterilized by soaking for 7 min in a 70% (v/v) ethanol. The seeds were then rinsed in sterile water and placed in a 30% (v/v) bleach solution for a further 20 min and then rinsed and resuspended in sterile water and stored at 4°C for 24 h. Seeds were germinated on 0.8% (w/v) agar containing 0.44% MS medium with 1% (w/v) sucrose, adjusted to pH 5.9 with KOH. Thiamine or TPP supplementation was at either 10 or 50 mg L^{-1}. The plates were placed in a growth cabinet at 22°C, 16 h light/8 h dark, light levels of 200 μmol m^{-2} s^{-1}. At 16 d after germination, seedlings were transferred to soil (Levington F2 compost), placed in the greenhouse, and watered with Hoagland solution. For supplementation experiments (data not shown) with Xu5P, G3P, and pyruvate, seeds were germinated in 96-well plates containing MS media and 1% (w/v) sucrose. A dilution series was used of between 31 and 100 μM Xu5P, G3P, and pyruvate.

For the analysis of photosynthesis, carbohydrates, and growth (Figures 3 to 5), freshly plated seeds were germinated on MS medium in a climate-controlled growth chamber at 22°C, photoperiod of 16 h light/8 h dark, and an irradiance of 200 to 250 μmol m^{-2} s^{-1}. After 12 d, seedlings were transferred to soil, and at 26 d, plants were moved to larger pots and placed in a controlled environment chamber at 25°C, with light levels of 250 μmol m^{-2} s^{-1} and a 16-h photoperiod.

Wild-type plants and TKox lines were grown at different light levels in greenhouse conditions. For the low-light experiment, shading was used to give 80 to 100 μmol m^{-2} s^{-1}, and for high light, plants were grown on the adjacent bench in the open at 500 to 2000 μmol s^{-1}. The temperature range used was between 28 and 35°C (data not shown). Plants subjected to different daylengths were grown in very short days 4 h light/20 h dark, short days 8 h light/16 h dark, and long days 16 h light and 8 h dark in the controlled environmental chamber with light level of 200 to 250 μmol m^{-2} s^{-1} and 27°C (data not shown). Plants were germinated on plates and transferred to soil as described in the paragraph above.

For the experiments in Figure 8, seeds of the wild type and the three TKox lines were grown on plates with or without thiamine (50 mg L^{-1}) plates. The plates were placed in a growth cabinet for 16 h light/8 h dark at light levels of 250 μmol m^{-2} s^{-1} and a temperature of 22°C. At 12 d after germination, plants were transferred to compost (Levington), and after 1 week, plants were transferred to larger pots and placed in long-day conditions in the greenhouse, with light levels of 600 to 1500 μmol m^{-2} s^{-1}. For plants receiving thiamine supplementation, this was applied every third day throughout the life cycle of the plants.

For the sampling of plant material at growth stages ST1 and ST2 (Figure 10), wild-type and TKox lines were germinated in compost (Levington F2 compost). Trays were placed in a controlled environment cabinet with 16 h light/8 h dark at light levels of 250 μmol m^{-2} s^{-1} and a temperature of 22°C. Samples were taken at the midpoint of the photoperiod when they read the following growth stages: ST.1 (cotyledons 3 mm at widest point across both and no first true leaf evident) and ST.2 (cotyledon fully expanded and 1st true leaf visible). All material was immediately snap frozen in liquid nitrogen and stored at −80°C for later analysis.

Primary root length was measured by positioning the plates vertically in the growth chamber. Shoot/rosette growth of younger plants (<26 d) was recorded by photographing the plants. Images were analyzed using the ImageJ software package (Image J version 1.45). For destructive harvest growth analysis, leaves and roots were separated from the plants and laid out flat, photographed, and analyzed using ImageJ, while dry weights were obtained by drying plant material at 60°C until a constant weight was obtained (~4 d).

RNA Extraction and Quantitative PCR Analysis

RNA was extracted from frozen leaf (100 mg) material using Tri-reagent (Sigma-Aldrich) according to the manufacturer's instructions. DNA was

degraded using a DNase1 kit (Invitrogen) following the manufacturer's instructions. Total RNA concentration and quality were determined using a nano-drop spectrophotometer. cDNA synthesis was achieved by adding 1 μM oligo(dT) primers, 200 μM deoxynucleotide triphosphate mix, and 1 μg RNA. Quantitative PCR (qPCR) amplification reactions were performed using Sensifast qPCR reagents (Bioline) and run in triplicate on 96-well plates on a Bio-Rad CFX96 qPCR system. Reactions were prepared in a total volume of 15 μL using 10 μL 2× Sensifast qPCR mix, 1.5 μL primer mix (10 μm), and 6 μL CDNA (0.25 ng total). The primers are listed in Supplemental Table 3. The cycling conditions were as follows: initial denaturation, 95°C, 2 min to activate the Taq polymerase, followed by 45 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 10 s, and extension at 72°C for 10 s. The amplification was followed by a melting curve analysis that ranged from 65 to 90°C with a temperature increase rate of 0.5°C. Baseline, threshold cycles (Cq values), and results were determined using the Bio-Rad CFX manager software (v3.1) according to the manufacturer's instructions. The qPCR reference genes used (elongation factor and actin) were taken from Schmidt and Delaney (2010). All other primers were produced in Primer3 on sequences from the NCBI database except those used to identify the alternate splicing of ThiC primers (Wachter et al., 2007). Confirmation of the correct PCR product being synthesized was done by running the qPCR samples on an agarose gel and from qPCR melting temperature analysis. To alleviate concerns about signal interference through the replication of genomic DNA, a sample of RNA was taken after DNase treatment (-RT control) but before the RT reaction. This -RT sample was used in a qPCR reaction with the reference primers, and if the cT (cross threshold) value was more than 10 cT values higher than in the corresponding RT-PCR reactions, then it was deemed not to interfere with the final result (Expósito-Rodríguez et al., 2008).

Thiamine Extraction and Determination

Thiamine and TPP were extracted as described (Kozik, 1995) with the following modifications. Leaf material (300 mg) was frozen in liquid nitrogen, crushed, ground in extraction buffer (50 mM potassium phosphate buffer, pH 7, 0.15 M NaCl, 1 mM phenylmethylsulphonyl fluoride [dissolved in DMSO], 1 mM EDTA, 1 mM DTT, and 1% polyvinylpyrrolidone), transferred to a 1.5-mL tube, and centrifuged at 4°C for 30 min at 14,000*g*. The supernatant (500 μL) was added to trichloroacetic acid (v/v 10%, 250 μL), mixed, and placed on ice for 10 min, then centrifuged at 4°C for 10 min at 6000*g*. The resulting supernatant (500 μL) was transferred to a fresh tube and the pH of the solution adjusted to ~ pH 3.0 using methanol.

Seeds (100 mg) were analyzed to determine thiamine content in the following conditions: dry or imbibed in water or in thiamine (10 mg L^{-1}). Seed produced from TKox plants that had been supplemented with thiamine (50 mg L^{-1}) throughout their life cycle (+/+) was also analyzed. Seeds were imbibed for 24 h at 4°C and then washed in water, dried, and then stored at −20°C until analysis. Dry seeds were also stored at −20°C.

Measurement of thiamine and thiamine pyrophosphate was performed using HPLC. Prior to HPLC analysis, thiamine was derivatized to thiochrome in alkaline conditions (Lu and Frank, 2008). Thiochrome formed by oxidation of thiamine emits blue fluorescence under UV light, which allows determination of individual thiamine and TPP concentrations. Hexacyanoferrate III was used as the derivatizing agent and was prepared by mixing 15% NaOH (w/v) and 1% potassium ferricyanide (w/v) in a 10:1 ratio. These solutions were kept for up to 1 week and stored at 4°C; for thiamine measurements, the two chemical solutions were mixed and used immediately. The derivatizing agent (25 μL) and thiamine plant extracts (40 μL) were pipetted into a 96-well plate, sealed, and placed in the HPLC Well Plate Auto Sampler (Agilent G1367). This technique provided samples that were stable for up to 24 h with <5% variation in fluorescence values.

Thiochrome products were analyzed using reverse phase chromatography and separated on a column (Phenomenex Luna C18; 150 × 4.6 mm, 5 μm protected with a 4.6-mm guard cartridge). The injection volume was 20 μL, the column temperature was 25°C, and the auto sampler temperature 10°C. Fluorescence excitation was at 375 nm, while emission was 435 nm. The mobile phases were (A) 20 mM Na_2HPO_4 + 5% methanol (pH 7) and (B) 20 mM Na_2HPO_4 + 50% methanol (pH 7). The protocol was 0 to 2 min of 100% phase (A) then from 2 to 12 min mobile phase (B) increased to 100%, which was held for a further 8 min. From 20 to 24 min, 100% of mobile phase (A) was used to prepare the column for the next sample.

Detection of Starch

Leaves from 3- to 4-week-old tobacco seedlings were bleached by boiling in 80% ethanol (v/v) for 20 min. The colorless leaves were then stained with a Lugol's solution (6 mM iodine, 43 mM KI, and 0.2 N HCl). The leaves were then destained using distilled water and photographed.

Measurements of Photosynthetic Gas Exchange and Chlorophyll Fluorescence

Photosynthesis (A) was measured as a function of internal CO_2 concentration (C_i), A/C_i response curves, using a portable gas exchange system (LI-COR 6400); leaf temperature was 25°C and leaf vapor pressure deficit was at ~1.1 ± 0.5 kPa. Leaves were initially stabilized in the cuvette at a saturating PPFD of 1200 μmol m^{-2} s^{-1} (provided by a red-blue light source) and 400 μmol mol^{-1} CO_2 concentration. Measurements of A and C_i were taken when A was stable. Subsequently, CO_2 concentration was decreased stepwise to 50 mol mol^{-1} before returning to 400 mmol mol^{-1} and then increased stepwise to 1600 mol mol^{-1} CO_2, and measurements recorded when A was stabilized. Values of A and C_i were calculated using the equations of (von Caemmerer and Farquhar, 1981).

Chlorophyll fluorescence imaging measurements were performed on tobacco seedlings grown in agar plates using a CF FluoroImager chlorophyll fluorescence imaging system (Technologica) (Barbagallo et al., 2003; Baker and Rosenqvist, 2004). The maximum quantum efficiency of PSII photochemistry (F_v/F_m) was determined in dark-adapted (15 min) seedlings from measurement of minimal fluorescence (F_o) obtained using a weak measuring pulse (<1 μmol m^{-2} s^{-1}) followed by an image of maximal fluorescence (F_m) obtained with a saturating pulse of 4800 μmol m^{-2} s^{-1} for 800 ms. Images of F_v/F_m were built using the following equation $F_v/F_m = (F_m\text{-}F_o)/F_m$. Images of PSII operating efficiency (F_q'/F_m'; $F_q' = (F_m' - F')/F_m'$) were obtained at an actinic light level of 300 μmol m^{-2} s^{-1} from steady state fluorescence images (F'), and images of F_m' capture immediately following the application of a saturating pulse (4800 μmol m^{-2} s^{-1} for 800 ms) at 5-min intervals for 15 min. The actinic light was then increased to 500 mmol m^{-2} s^{-1} and the measurements repeated. F_q' was calculated from the difference in fluorescence between F_m' and F', where F' is the steady state fluorescence under actinic light. F_m' is maximum fluorescence in the light-adapted state. For each measurement, three plants were measured simultaneously (two transgenic and one wild type). The effect of light on the F_q'/F_m' was determined on the same leaves as infrared gas exchange measurements were collected. At each light level, fluorescence was left to stabilize for 3 min, after which F' and F_m' images were capture and used to determine F_q'/F_m' as described above.

Soluble Sugar and Starch Determination

The soluble carbohydrates (sucrose, fructose, and glucose) and starch were extracted from leaf tissue over a diurnal cycle. The leaf discs were incubated in 80% (v/v) ethanol for 30 min at 80°C and then washed four times with ethanol 80% (v/v). Sucrose, glucose, and fructose were measured from the ethanol extract using an enzyme-based protocol (Stitt and Quick, 1989), and the starch contents were estimated from the ethanol-insoluble pellet according to Stitt et al. (1978), with the exception that the samples were boiled for 1 h and not autoclaved.

Metabolite Analysis and Enzyme Activities

Metabolite analysis was performed as described previously (Henkes et al., 2001). Enzymes were extracted as described (Nunes-Nesi et al., 2007). Activities of AGPase, NADP-GAPDH, and TK were measured as described by Gibon et al. (2004) and the activity of FBPAldolase as described by Piques et al. (2009).

Protein Extraction and Immunoblotting

Leaf discs were ground in liquid nitrogen and protein quantified (Harrison et al., 1998). Samples were loaded on an equal protein basis, separated using 12% (w/v) SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed using polyclonal antibodies raised against tobacco plastid TK as described by Henkes et al. (2001). PRKase polyclonal antibodies were a gift from M Salvucci, and antibodies against DXS were from Patricia Leon. Proteins were detected using horseradish peroxidase conjugated to the secondary antibody and ECL chemiluminescence detection reagent (Amersham).

Statistical Analysis

All statistical analyses were performed using one-way ANOVA, Tukey's post hoc test in R v.2.6.2 (http://www.r-project.org). Significant differences between transgenic lines and the wild type for the different parameters evaluated are reported with asterisks (*$P > 0.05$, **$P < 0.01$, and ***$P < 0.001$; ns, nonsignificant) in the figures. In tables, significant differences ($P < 0.05$) between transgenic lines and the wild type for the different parameters evaluated are reported as different letters unless indicated otherwise. Same letter indicates nonsignificant difference.

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under accession numbers At3g60750 (*TKL1*) and AT2G45290 (*TKL2*).

Supplemental Data

Supplemental Figure 1. Expression of *TKL2* and *TKL1* at Different Developmental Stages of Arabidopsis.

Supplemental Figure 2. Growth and Chlorotic Phenotypes of TKox Plants after Transfer to Soil.

Supplemental Figure 3. The Levels of Transketolase and FBPaldolase Protein in Wild-Type and TKox Lines.

Supplemental Figure 4. Starch Content of TKox Lines 1 and 4 and Wild-Type Plants Determined Using Iodine Staining.

Supplemental Figure 5. Fluorescence Image Analysis Shows Functional Complementation of the TKox Phenotype by TPP.

Supplemental Figure 6. Thiamine Supplementation Rescued Root Growth in the TKox Lines.

Supplemental Figure 7. Effect of TPP on TKox Tobacco Plants Grown in Soil.

Supplemental Figure 8. Timing of Thiamine Supplementation Is Critical to Rescue TKox Lines.

Supplemental Figure 9. Dry Weight and Total Leaf Area of TKox and Wild-Type Plants.

Supplemental Figure 10. Imbibition of Seeds of TKox in Water Supplemented with Thiamine Is Sufficient to Complement the Growth and Chlorotic Phenotype.

Supplemental Table 1. Enzyme Activities, Pigment, and Metabolite Contents in TKox Plants.

Supplemental Table 2. Statistical Analysis for Data Presented in Figure 4.

Supplemental Table 3. List of Primers Used in the qPCR Analysis

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

C.A.R. conceived the project and together with S.C.L. designed the approach. S.C.L., M.K., and S.F. performed the experiments. M.S. provided expertise and access to analysis of metabolites. Y.G. and R.S. provided input into the data collection analysis of the metabolites and enzyme activities. T.L. provided expertise on photosynthesis. C.A.R., S.C.L., M.K., T.L., and S.F. analyzed the data. C.A.R. wrote the article with input from T..L, M.K., and S.F. S.C.L., M.S., Y.G., and R.S. commented on the article.

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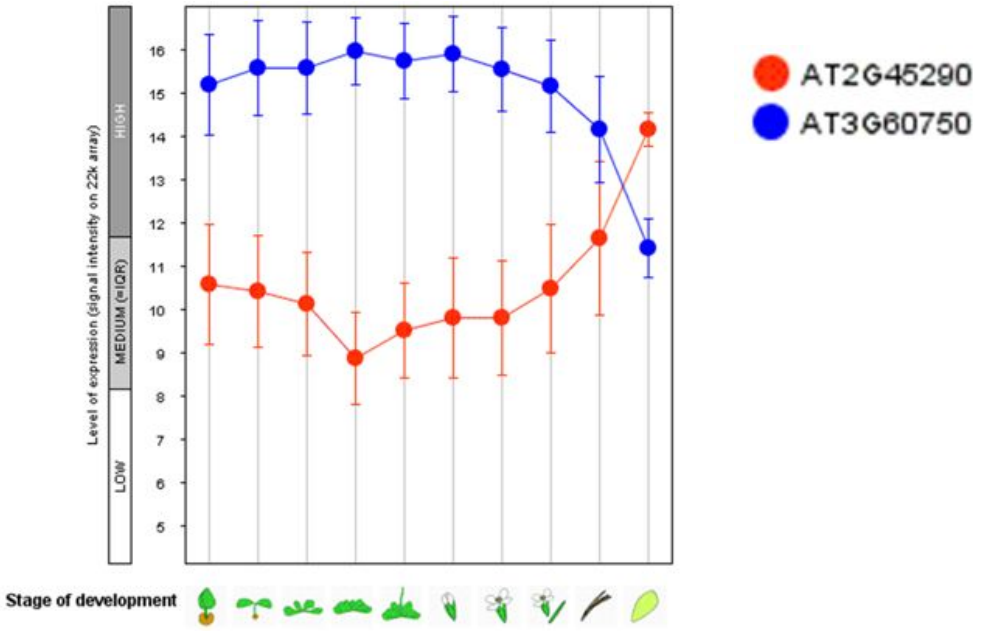
A



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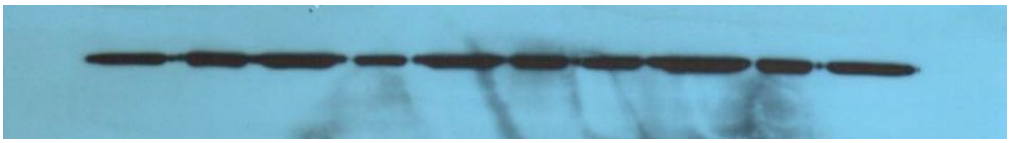
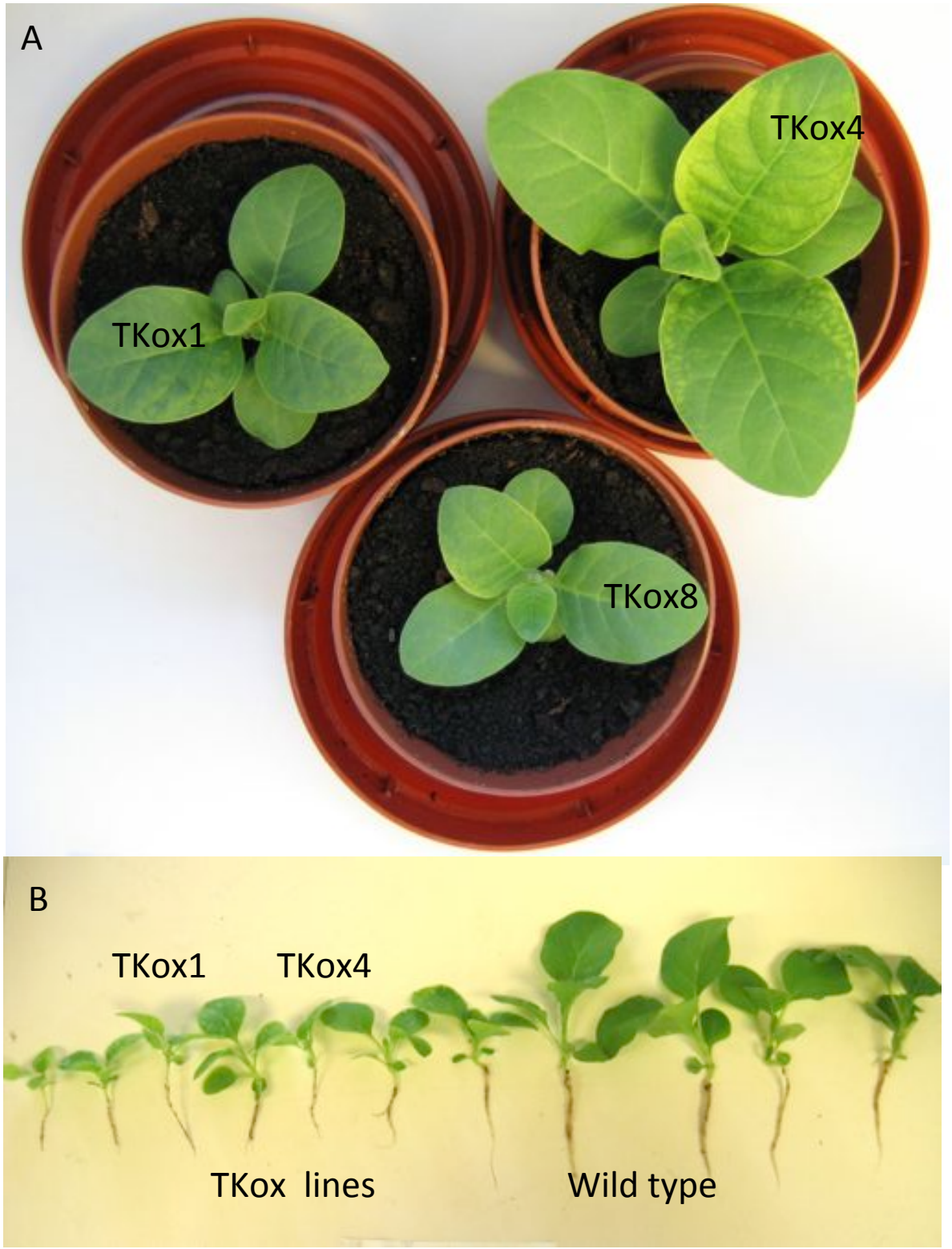
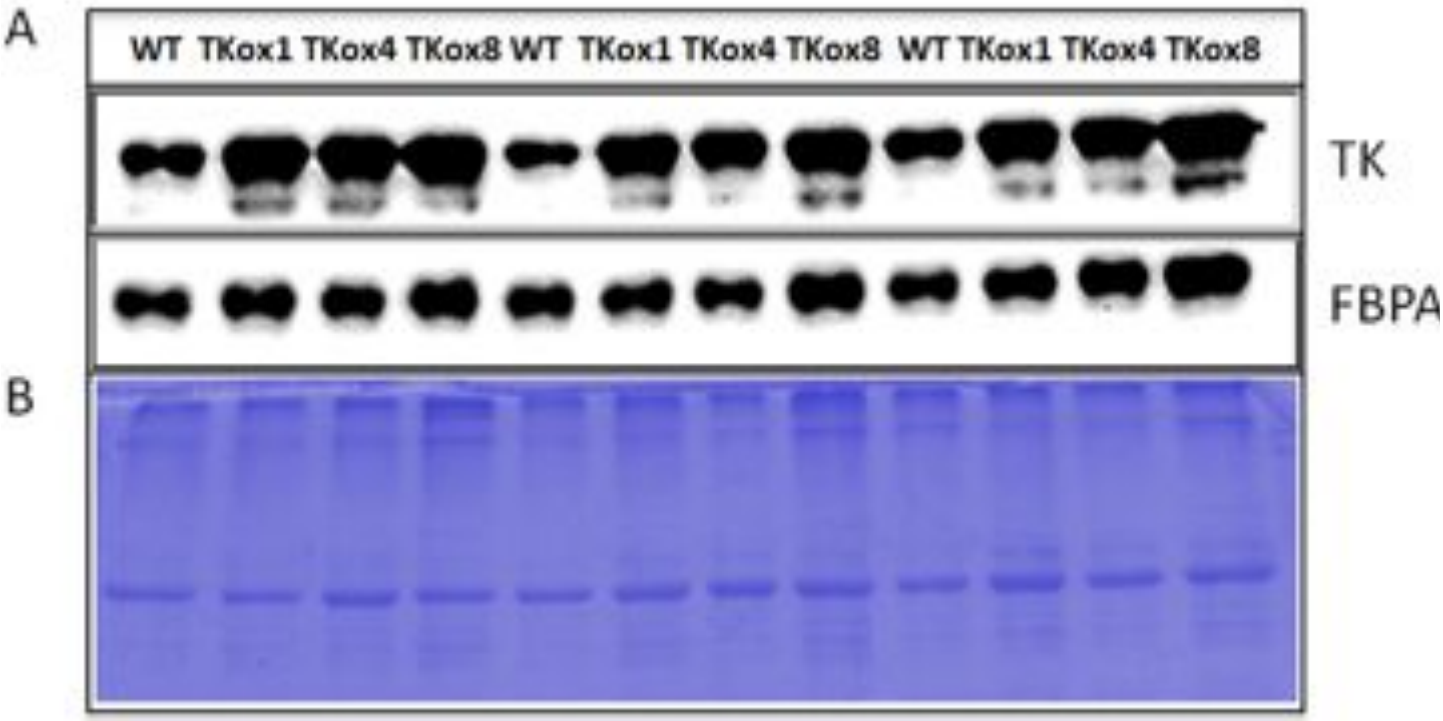
B



Supplemental Figure 1. Expression of the Arabidopsis TKL1 and TKL2 genes at different developmental stages. A. Expression of RNA transcripts for the TKL1 and TKL2 genes in germinated seed, seedlings, young rosettes, developed rosettes, at bolting, young flowers, developed flowers, flowers and siliques, mature siliques and senescing leaves. (Image created with Genevestigator, August 2014, https://genevestigator.com/gv/plant.jsp.) Error bars indicate the SD of the results from the experimental database. **B.** Immunoblot analysis of primary transformants overexpressing transketolase. Proteins were extracted from two consecutive newest fully expanded leaves of each of three T0 transformant TKox plants (-1, -4 and -8). Proteins were separated using SDS-polyacrylamide gel electrophoresis and blotted onto nylon membrane and subjected to immunobloting. TK protein levels were revealed using polyclonal antibodies raised against native TK (Henkes et al., 2001).



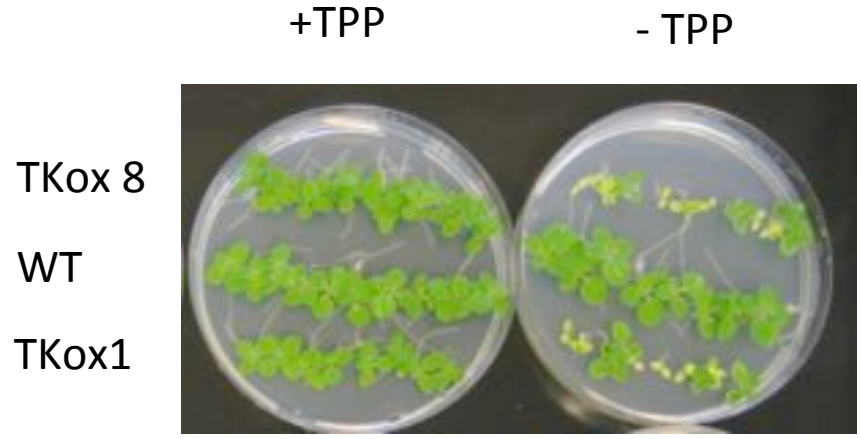
Supplemental Figure 2. Growth and chlorotic phenotypes of TKox plants after transfer to soil. TKox plants after transfer to soil display (**A**) a chlorotic phenotype and (**B**) reduced growth rates of shoots and roots. Plants were grown in controlled environment conditions (16 h dark/8 h light) for 5 weeks with light levels of 200-250 μmol m^{-2} s^{-1}.



Supplemental Figure 3. The levels of transketolase (TK) and FBPaldolase (FBPA) protein in WT and TKox lines. Protein was extracted from the newest fully expanded leaf of WT and TKox lines and the resulting samples were separated on an 12% acrylamide gels and analysed by (**A**) immunoblotting using polyclonal antibodies raised against native TK and synthetic peptides of FBPA and (**B**) staining using Coomassie Blue . The results shown are for three biological replicates for each of the TKox transgenic lines and WT plants.

Supplemental Figure 4. Starch content of TKox lines 1 and 4 and WT plants determined using iodine staining. Plants were grown in (**A**) long days (16 h light 8 h dark) and (**B**) short days (8 h light 16 h dark) with 200-250 µmol m^{-2} s^{-1} light at 25 ºC. Leaves were sampled from one month old plants at the end of the night and subjected immediately to iodine staining.

A



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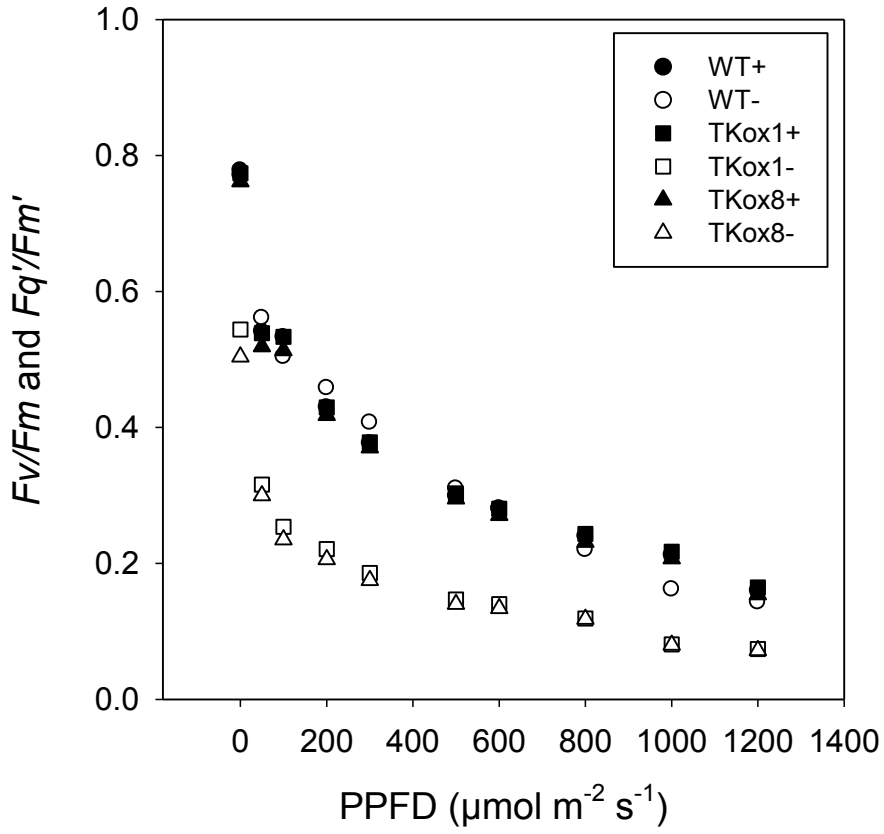
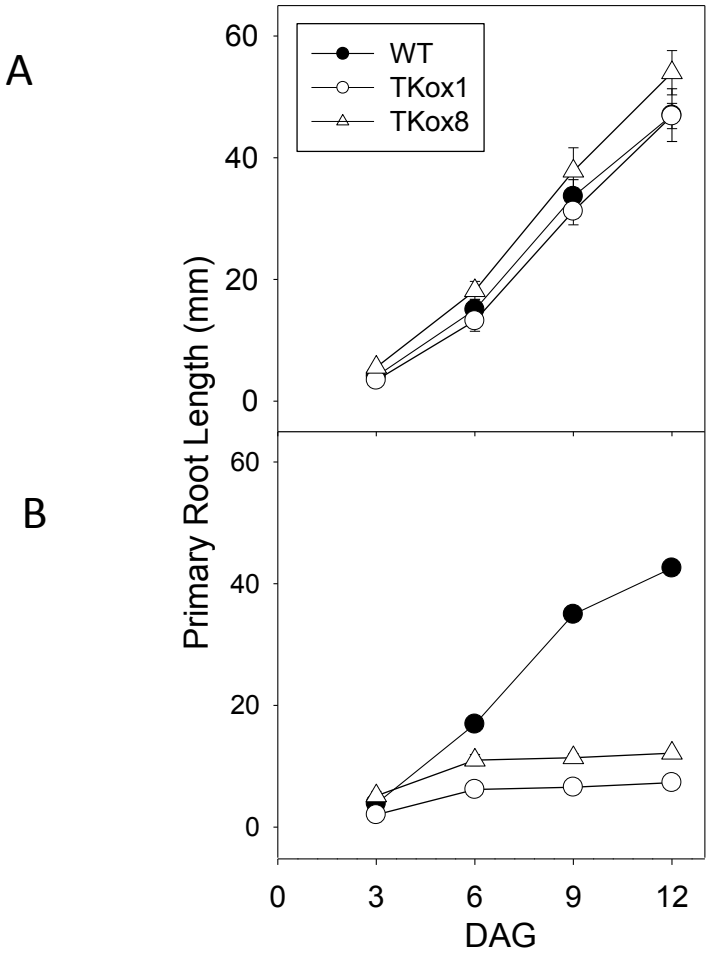
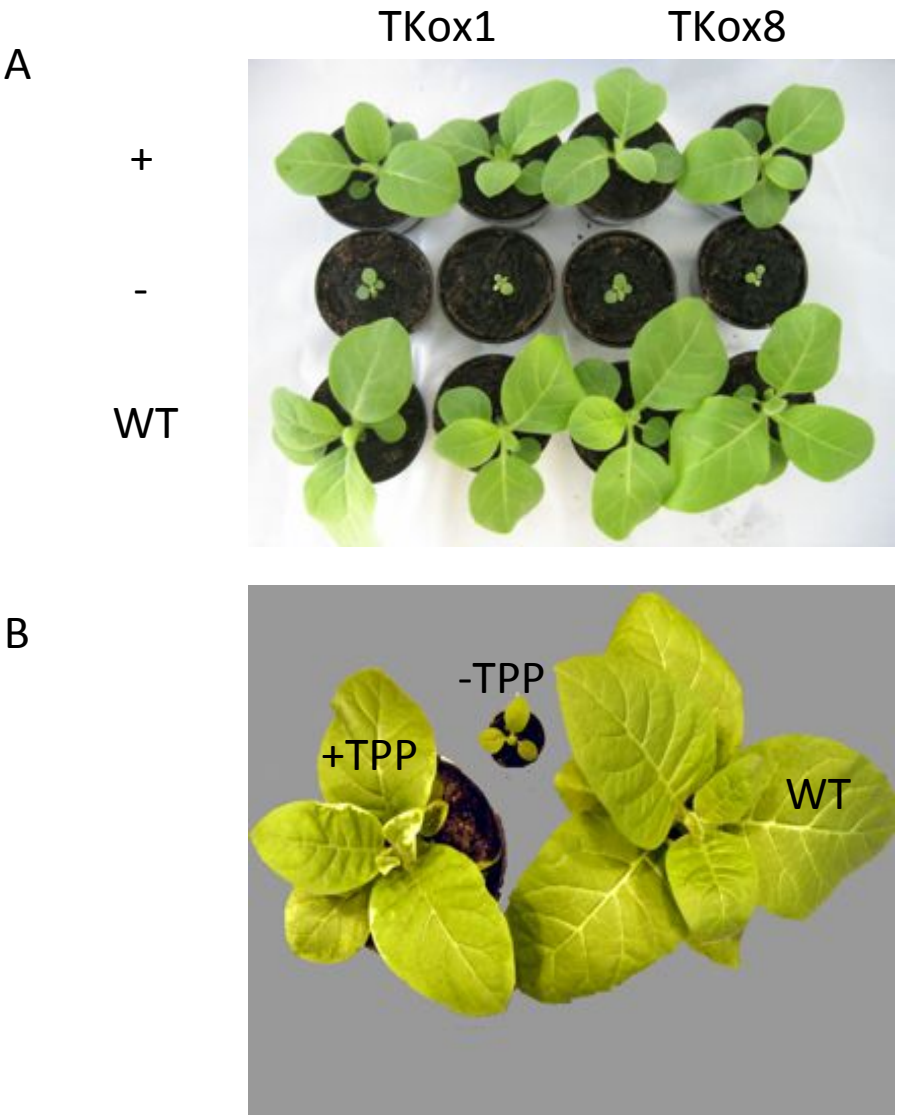


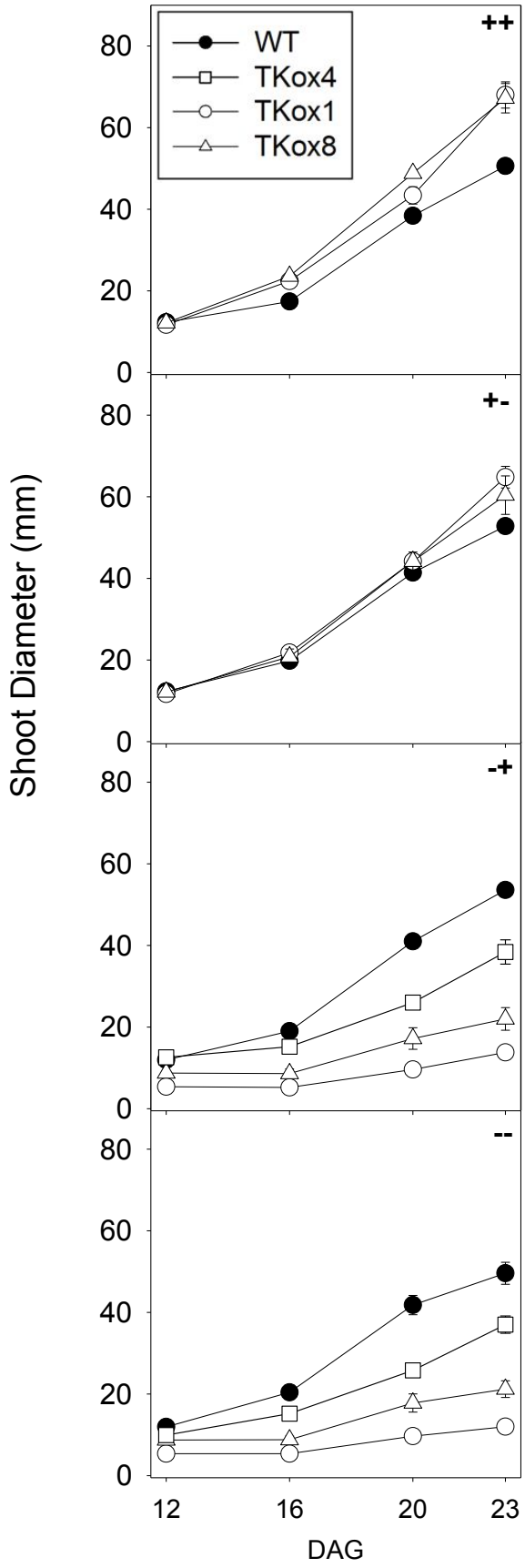
Supplemental Figure 5. Fluorescence image analysis shows functional complementation of the TKox phenotype by TPP
Functional complementation by TPP. A. Appearance of the TKox lines 1 and 8 and WT plants grown in MS media for 12 days with or without TPP (10 mg L^{-1}). **B.** The chlorophyll fluorescence parameters Fv/Fm and Fq'/ Fm' were determined from chlorophyll fluorescence imaging of a duplicate set of dark-adapted TKox and WT plants as shown in Figure 7.



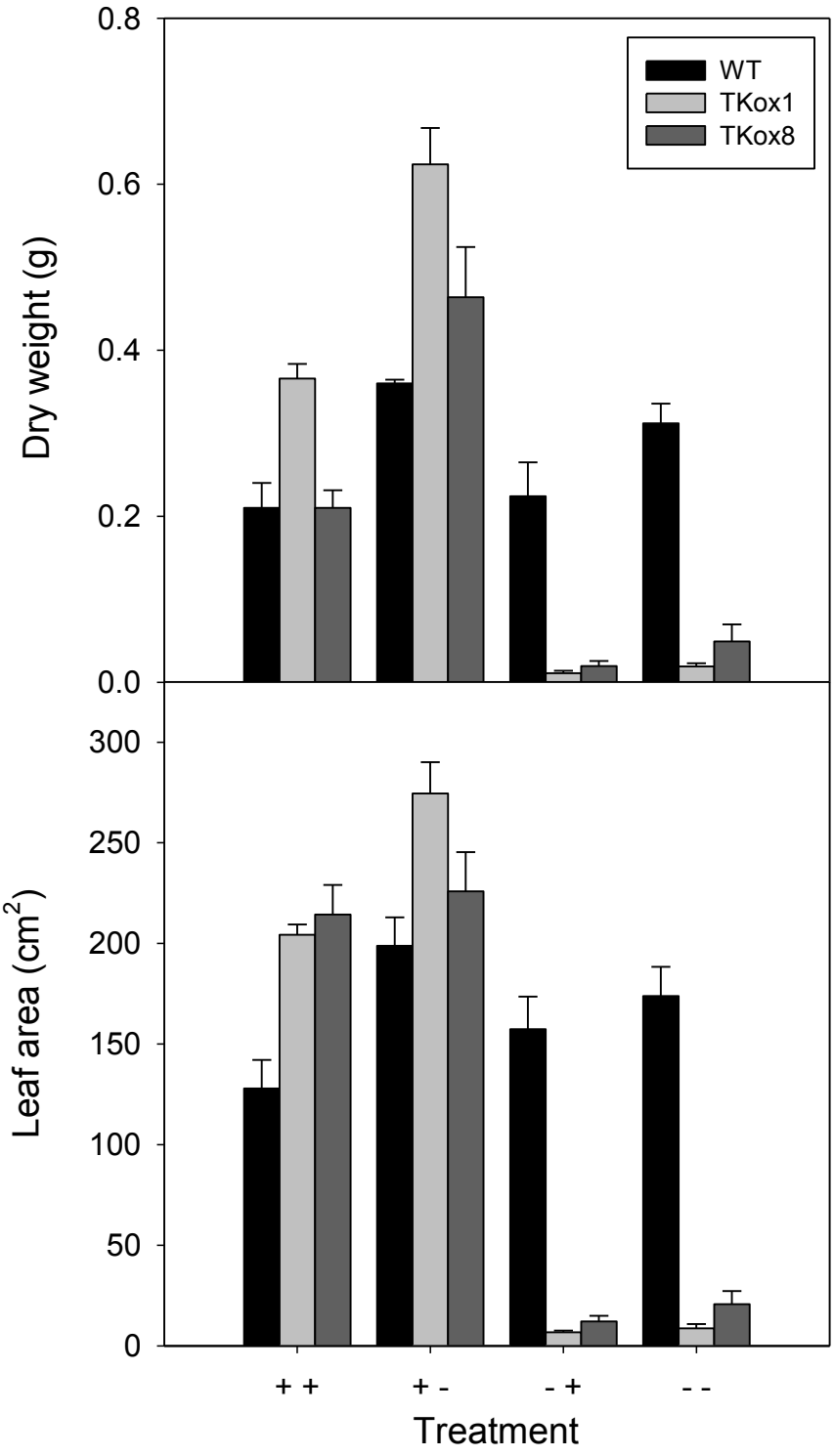
Supplemental Figure 6. Thiamine supplementation rescue d root growth in the TKox lines. TKox (lines 1 and 8) and WT plants were germinated in MS media (A) alone or (B) containing thiamine (50 mg L^{-1}) and grown for 12 d and the length of the primary root determined. Results are the mean ± SE (n = 5).



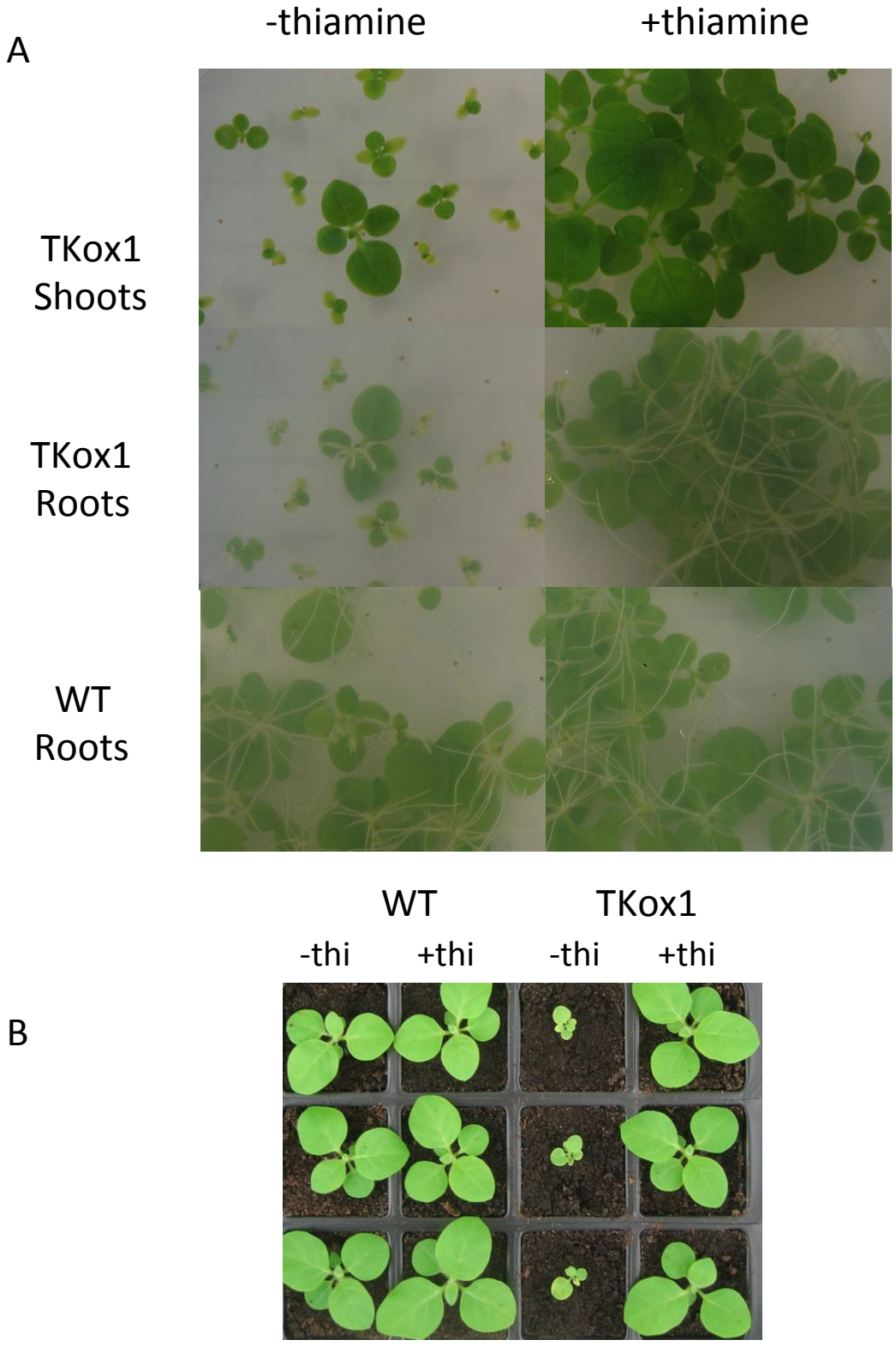
Supplemental Figure 7. Effect of TPP on TKox tobacco plants grown in soil. TKox (lines -1 and -8) and WT plants were grown in media supplemented with (+) or without (-) 10 mg L^{-1} TPP and then transferred to soil 12 d after germination, grown in a controlled environmental chamber with light levels of 200-250 mmol m^{-2} s^{-1} with no further supplementation. **A**. three weeks after germination, TKox lines (-1 and -8, top two rows) and WT (bottom row) and **B**. five weeks after germination (line TKox-1 only).



Supplemental Figure 8. Timing of thiamine supplementation is critical to rescue TKox lines. TKox and WT plants were germinated in media containing thiamine (50 mg L^{-1}) and then transferred to soil 12 d after germination (DAG) and watered with thiamine (+/+) or without (+/-). A second set of plants were germinated without thiamine and watered with thiamine (-/+) or without (-/-). Plants were transferred to soil 12 DAG. The shoot diameter determined at five time points after transfer to soil. For TKox-4 only +/+ and -/- data are shown. Error bars indicate the s.e (n=4).



Supplemental Figure 9. Dry weight and total leaf area of TKox and WT plants. Plants were from the same set as described for Supplemental Figure 8. Destructive harvest was at 34 days after germination and leaf area and shoot dry weight determined. TKox (lines 1 and 8) and WT plants germinated in media containing thiamine (50 mg L^{-1}) and then transferred to soil 12 days after germination and watered with thiamine (+/+) or without (+/-). Plants germinated without thiamine and watered with thiamine (-/+) or without (-/-). Results are the mean ± SE (n = 5).



Supplemental Figure 10. Imbibition of seeds of TKox in water supplemented with thiamine is sufficient to complement the growth and chlorotic phenotype. Seeds of WT and TKox-1 plants were imbibed in water or water plus thiamine (50mg L^{-1}). Following rinsing with water, seeds were place on MS media with no further thiamine. **A**. two weeks growth on MS media. **B**. Seedlings were transferred to soil and growth recorded after 20 d.

Category	Analysis	WT (n=4)	TKox4 (n=6)	TKox1 (n=4)
Enzyme activity (µmol g FW^{-1} min^{-1})	Shikimate dehydrogenase	1.2 ±0.18	1.3 ±0.12	1.4 ±0.57
	Phosphoglucose isomerase (Total)	7.0 ± 0.48	6.7 ± 0.27	8.0 ± 0.45
	Phosphoglucose isomerase (cytoplasmic)	5.1 ± 0.44	4.9 ±0.24	6.0 ±0.24
	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	14.3 ±0.12	15.5 ±0.8	18.4 ±0.89
	Glucose-6-phosphate dehydrogense	0.76 ±0.52	0.84 ±0.38	0.88 ±0.65
	F-6-phosphate aldolase	26.1 ±0.15	27.5 ±0.19	33.0 ±0.23
	F-6-phosphate aldolase (cytoplasmic)	0.70 ±0.40	0.70 ±0.53	0.79 ±0.33
	ADP-glucose pyrophosphorylase	3.0 ±0.31	3.0 ±0.27	3.8 ±0.35
	Transketolase	27.3 ±0.18	48.0 ±0.49	59.3 ±0.5
Phenylpropanoids	Nicotine (µmol gFW^{-1})	4.6 ±0.2	5 ±0.6	6.2 ±0.3
	Chlorogenic acid (µmol gFW^{-1})	2.9 ±0.16	2.6 ±0.41	3.1 ±0.15
	Rutine (nmol gFW-1)	239 ±75	221 ±56	319 ±42
	p-Coumaric acid (nmol gFW^{-1})	39 ±3.5	46 ±4.5	33 ±7.4
	Benzoeic acid (nmol gFW^{-1})	10.5 ±0.65	11.1 ±0.46	13.2 ±1.3
Pigments (µmol gFW^{-1})	Chlorophyll A	1551 ±116	1316 ±154	1704 ±66
	Chlorophyll B	161 ±12.2	163 ±8.6	161 ±6.5
	Chlorophyll A&B	1712 ±126	1479 ±162	1866 ±70
	Ratio Chlorophyll A/B	9.6 ±0.5	7.9 ±0.7	10.6 ±0.4
Amino acids (µmol gFW^{-1})	Aspartic acid	4.7 ±0.7	5.3 ±0.4	6.8 ±0.9
	Glutamic acid	3.9 ±0.6	4.3 ±0.5	6.9 ±0.8
	Asparagine	0.5 ±0.1	0.67 ±0.13	0.6 ±0.08
	Serine	1.2 ±0.2	1.38 ±0.1	1.61 ±0.3
	Glutamine	7.2 ±1.9	5 ±0.7	5.17 ±0.7
	Glycine	0.72 ±0.2	0.56 ±0.1	0.95 ±0.2
	Threonine	0.8 ±0.1	1 ±0.1	1.3 ±0.2
	Citrulline	0.15 ±0.04	0.12 ±0.02	0.15 ±0.03
	Alanine	1.7 ±0.3	2 ±0.2	2.5 ±0.5
	Arginine	0.5 ±0.1	6 ±0.1	0.7 ±0.2
	GABA	0.4 ±0.05	0.5 ±0.08	0.6 ±0.06
	Tyrosine	0.14 ±0.04	0.11 ±0	0.15 ±0.01
	Valine	0.3 ±0.05	0.2 ±0.02	0.3 ±0.03
	Phenylalanine	0.4 ±0.1	0.4 ±0.03	0.4 ±0.06
	L-Isoleucine	0.1 ±0.02	0.1 ±0.01	0.1 ±0.02
	Leucine	0.3 ±0.07	0.3 ±0.02	0.3 ±0.03
	Lysine	0.13 ±0.02	0.15 ±0.02	0.2 ±0.03

Supplemental Table 1. Enzyme activities and pigment and metabolite contents were determined in green leaf tissues of five-week-old transgenic TKox and wild-type plants grown in greenhouse conditions under light levels of between 600 to 1500 µmol m^{-2} s^{-1}, 25°C. Values are given as mean ± SE.

GLUCOSE

	TKox1	TKox4	TKox8
9:00 am	*		
12:00 pm			
6:00 pm	*	*	
12:00 am	*	*	*
6:00 am	*		
9:00 am			

FRUCTOSE

	TKox1	TKox4	TKox8
9:00 am	*		
12:00 pm	*	*	
6:00 pm	*		*
12:00 am	*	*	*
6:00 am	*		*
9:00 am			

SUCROSE

	TKox1	TKox4	TKox8
9:00 am			
12:00 pm			
6:00 pm	*		
12:00 am			
6:00 am			
9:00 am			

STARCH

	TKox1	TKox4	TKox8
9:00 am	*		*
12:00 pm	*		
6:00 pm		*	
12:00 am	*	*	*
6:00 am	*	*	*
9:00 am			*

Supplemental Table 2. Statistical analysis for data presented in Figure 4. Diurnal carbohydrate accumulation in TKox and WT tobacco plants. Whole plants were harvested at 6 time points over a diurnal cycle and levels of glucose, sucrose, and starch determined. Each value represents the mean of four measurements ± SE. Statistical comparison was made using a one way ANOVA and Tukey post hoc test, * indicates significance at P =< 0.05 compared to WT.

Name	Accession number	Primer Direction	Sequence	Produce size (BP)	Melt temp.
Elongation Factor	AF120093.1	F	TGAGATGCACCACGAAGCTC	51	80
		R	CCAACATTGTCACCAGGAAGTG		
Actin	X69885	F	CCTGAGGTCCTTTTCCAACCA	57	78
		R	GGATTCCGGCAGCTTCCATT		
ThiC 1	ABQ66370.1	F	CGTAACTGGTAAAGTTGTTGTCATGT	83	83
		R	TGCATTTCTACAAGAGGTTGTTTG		
ThiC 2	ABQ66370.1	F	TGAAAAGCTAAAGCTTCATAGATTGA	135	81.5
		R	ATGAAGCTCCTACACCCTCCT		
ThiC 3	ABQ66370.1	F	AAGTGTTAGGGGTGCCTGTG	130	83
		R	AAAATGCACACTCCCTACGC		
TH1	CHO_OF4997xk18r1.ab1	F	GCTCTGTTTGATAGGGAATGTGT	63	78
		R	CTCCTTGAGCACCTCGAGTAA		
THI1	AY220080.1	F	ACGCTGCTGAGGATGCTATT	110	78
		R	AGTTGGTCCCATTCTTGGTG		
TPK	EU161633	F	CTTGGTTGCTTGACCCAAAT	123	82.5
		R	ACTGGGGTGGCTTAGGATCT		
DXS	ACF60511.1	F	AGGTTTCGGGTCTCATGTTG	147	80.5
		R	GATGGCGTAAGACCAGCTTC		
NATIVE TK	EU647214.1	F	CATGGCTCTGCCTCTTCTTC	81	80
		R	GGTGGTGATATTGGGATTGG		
A.T PTK	AT3G60750	F	TATCGCTCAATCCATGACCA	98	83.5
		R	CCACAATCGACGAATCAGTG		
TK1	DQ198165.1	F	TACTTACCCTTGGTTGCTGG	103	79.5
		R	GGAGATAACGGTGGAAACC		
PDH	SAL_US030xl18f1.ab1	F	GAGTTCGTTAGCGCTAGTTTCG	61	82.5
		R	GTTCACCGGCCACAAGAT		
ALS	CHO_OF318xf21f1.ab1	F	TTGTAACCCCTTCGAGCAAA	64	76.5
		R	TTGTTGGGGATGAAAGTGGT		
BCKDC	CHO_OF4579xp06r1.ab1	F	ATTTTCTGAATCTGGCAATGG	64	77.5
		R	CGGCCAACAAACGAAATAGA		
KGDH	CHO_OF321xj05f1.ab1	F	CCACCACATCCTTAATTCTTCTC	65	77.5
		R	TGATCATAGGCTGATTGATGGA		
PDC	X81855.1	F	GCTGATTCTTCCGGTTATGC	68	80.5
		R	GGATGTTGTTCTGCCACTAGC		
PRK	EB699778.1	F	TGTAGAGAGCCATTTGAGCAAC	61	77.5
		R	CATTTGTTGAGTAACTTCACCATAGAA		
SBPase	CN746587.1	F	CGCTTAATGTTTCAAGGACCA	66	77.5
		R	CCAGACTATCACCGATCTCACA		

Supplemental Table 3. List of primers used in the q-PCR analysis. The qPCR reference genes used (elongation factor and actin) were taken from (Schmidt and Delaney, 2010) all other primers were produced in Primer3 on sequences from the NCBI database except those used to identify the alternate splicing of ThiC primers (Wachter et al., 2007).

Overexpression of Plastid Transketolase in Tobacco Results in a Thiamine Auxotrophic Phenotype

Mahdi Khozeai, Stuart Fisk, Tracy Lawson, Yves Gibon, Ronan Sulpice, Mark Stitt, Stephane C. Lefebvre and Christine A. Raines

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