The effect of increased plastid transketolase activity on thiamine metabolism in transgenic tobacco plants

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Summary

Transketolase is a TPP dependent enzyme that affects the availability of intermediates in both the Calvin cycle and non-oxidative pentose phosphate pathway. Previous studies have indicated that changes to the activity level of transketolase can limit growth and development as well as the production of isoprenoids, starch, amino acids and thiamine. The overall aim of this project was to further advance the understanding of the mechanism linking increased TK activity and thiamine metabolism. *Nicotiana tabacum* mutants with increased total transketolase activity ~ 2 to 2.5 fold higher than WT plants were shown to have a reduced growth and chlorotic phenotype. In seedlings, these phenotypes were attributed to a reduction in seed thiamine content. Imbibition of TKox seeds in a thiamine solution produced plants that were comparable to WT plants. However, the chlorotic but not growth phenotype was found to return unless the plants underwent irrigation with a thiamine solution indicating that TKox plants are unable to produce sufficient quantities of thiamine to meet demand. Furthermore, the application of deoxy-xylulose-5-phosphate was also found to be able to partially complement the phenotype suggesting that flux from the C_3 cycle into the non-mevalonate pathway is being affected. Analysis of thiamine and TPP levels demonstrated that TKox plants were deficient in thiamine but not TPP in the majority of cases. In plants that had begun to flower, TKox lines had reduced thiamine and TPP levels in the 20^{th} fully open leaf compared to the same leaf in WT plants. Furthermore, sampling of leaf tissue from both WT and TKox seedlings at the same developmental stage indicated that high levels of TK protein may lead to the accumulation of TPP in these areas causing a reduction in the levels of thiamine and TPP in the rest of the plant thereby limiting growth and development.

ABBREVIATIONS

ABA	abscisic acid
AIR	5-amino-imidazole-ribonucleotide
ALD	aldolase
ALS	acetolactate synthase
APS	ammonium per sulfate
ATP	adenosine triphosphate
At-TKL	*Arabidopsis thaliana* transketolase
BCA	branched amino acid metabolism
BCAA	branched chain amino acid
BCKDH	branched chain ketoacid dehydrogenase
C_3	Calvin Benson cycle
CaMV	cauliflower mosaic virus
DMADP	dimethylallyl diphosphate
DMSO	dimethyl sulfoxide
DOXP	1-deoxy-d-xylulose 5-phosphate
DTT	dithiothreitol
DXR	1-deoxy-d-xylulose-5-phosphate reductoisomerase
DXS	deoxyxylulose 5-phopshate synthase
E4P	erythrose-4-phosphate
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
F6P	fructose-6-phosphate
G3P	glyceraldehyde 3-phosphate
GA	gibberellic acid
GDH	glycerol-3-phosphate dehydrogenase
GTP	guanosine-5'-triphosphate
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HET-P	5-(2-hydroxyethyl)-4-methylthiazole phosphate
HET-PP	5-(2-hydroxyethyl)-4-methylthiazole pyrophosphate
HMP-P	hydroxmethyl pyrimidine phosphate
HMP-PP	4-amino-2-methyl-5-hydroxymethylpyrimidine pyrophosphate
HPLC	high performance liquid chromatography
IDP	isopentenyl diphosphate
IRGA	infrared gas analyzer
KGDH	ketoglutarate dehydrogenase
MEcDP	methylerythritol cyclodiphosphate
MEP	2-c-methyl-d-erythritol 4-phosphate
MS	murashige and skoog medium
NAD+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)

NADP	nicotinamide adenine phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
OPPP	oxidative pentose phosphate pathway
ORF	open reading frame
PBS	phosphate buffered saline
PDC	pyruvate decarboxylase
PDH	pyruvate dehydrogenase
PEP	Phosphoenolpyruvic acid
PMSF	phenylmethanesulfonyl fluoride
PPFD	photosynthetic photon flux density
PRK	phosphoribulokinase
PVDF	polyvinylidene fluoride
R5P	ribose-5-phosphate
ROS	reactive oxygen species
RPI	ribose-5-phosphate isomerase
RuBisCO	ribulose-1,5-bisphosphate carboxylase oxygenase
RuBP	ribulose 1,5-bisphosphate
SAM	s-adenosyl-methionine
SBPase	sedoheptulose-1,7-bisphosphatase
SDS	sodium dodecyl sulfate
TAE	TRIS-base, acetic acid and edta
TBPs	thiamine binding proteins
TCA	tricarboxylic acid
TCAA	trichloroacetic acid
Temed	tetramethylethylenediamine
TH1	hydroxymethyl pyrimidine kinase
Thi	thiamine thiazole synthase
Thiamine	vitamin b1
ThiC	hmp-p-synthase
TK	transketolase
TMP	thiamine mono-phosphate
TPI	triose-phosphate isomerase.
TPK	thiamine pyro-phosphokinase
TPP	thiamine pyro-phosphate
Tween-20	polysorbate 20
UTR	untranslated region
Xu5P	xylulose-5-phosphate

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CHAPTER 1

INTRODUCTION

Introduction

To supply sufficient food for an increasing world population it is essential that crop yields are increased (Ainsworth and Ort, 2010). The use of biotechnology and the manipulation of enzymes in the Calvin cycle is one way that has been identified to achieve this (Raines, 2011) (Stitt and Quick, 1989) (Sonnewald et al., 1994).The Calvin cycle is the primary pathway of carbon fixation in plants and provides a number of intermediates that are essential for the function of a number of connected pathways. Results from Calvin cycle enzyme manipulation studies have often found that yields are only increased if atmospheric CO_2 concentration and/or light levels are elevated (Lefebvre et al., 2005). One of the enzymes that was found to increased yields under saturating light and CO_2 levels was sedoheptulose-1,7-bisphosphatase (SBPase). This provided evidence that the over-expression of a single enzyme in the Calvin cycle can result in an increase in both carbon fixation and growth (Dunford et al., 1998; Lefebvre et al., 2005). The Calvin cycle enzyme, transketolase (TK) has been shown to have a negative effect on both photosynthesis and growth in antisense tobacco plants (Henkes et al., 2001)However the effect on metabolism was shown to be unique to TK when compared to other antisense studies (Haake et al., 1998; Harrison et al., 1998; Haake et al., 1999). This evidence suggested that an increase in the activity level of TK may result in an increase in photosynthesis and yield.

To test this hypothesis, tobacco TK sense plants (*Nicotiana tabacum* L.cv. Samsun) were produced (Lefebvre, 2005). Surprisingly these plants exhibited a slow growth and chlorotic phenotype. It is known that TK requires a phosphorylated form of thiamine known as thiamine pyro-phosphate (TPP) as well as a divalent metal ion to enable its activity (Kochetov, 1982). Interestingly, both the growth and chlorotic phenotypes were rescued by supplying the plants with an exogenous thiamine source

at germination (Khozaei, 2010). This result suggested that increased TK activity impacted on the availability of thiamine in the plant. The aim of the study in this thesis was to further analyse the role increased TK activity has on thiamine metabolism and its effect on plant development.

1.1 The Calvin cycle and pentose phosphate pathway.

The Calvin cycle can be divided into three main stages; carboxylation, reduction and regeneration (Figure.1.1). In the first stage, carboxylation, the enzyme ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) catalyses the fixation of atmospheric CO_2 to the 5 carbon acceptor molecule ribulose-1,5-bisphosphate (RuBP) creating two molecules of 3-phosphoglycerate. In the reduction stage, 3-phosphoglycerate is phosphorylated by 3-phosphoglycerate kinase using adenosine tri-phosphate (ATP); the products of this reaction are then reduced by the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using reduced nicotinamide adenine dinucleotide phosphate (NADPH). This produces two molecules of glyceraldehyde-3-phosphate (G3P) per carbon molecule fixed in the initial carboxylation reaction. This can be exported for use in the production of isoprenoids via the 2-C-methyl-d-erythritol 4-phosphate pathway (MEP) which provides precursors for the synthesis of a large number of compounds including isoprenoids, hormones and the side chains of chlorophylls (Phillips et al., 2008).

The third and final stage of the cycle is regeneration of the CO_2 acceptor molecule RuBP through a complex set of reactions that also produce a number of secondary pathway intermediates. One of these secondary pathway intermediates is erythrose-4-phosphate (E4P) which provides a link between the metabolism of carbohydrates and

the biosynthesis of aromatic compounds (Herrmann and Weaver, 1999), whilst R5P which is a product of the TK reaction, is utilized in the synthesis of nucleotides and thiamine.

In addition to TK's role in the Calvin cycle it also functions in the pentose phosphate pathway (PPP) (Figure.1.2). However, unlike the Calvin cycle which utilises CO_2, NADPH and ATP to produce hexose sugars, the PPP uses hexose substrates to produce NADPH and pentoses whilst releasing CO_2 in the process. The PPP also produces precursors for various biosynthetic processes. These include R5P which is used in the synthesis of nucleotides and E4P that is used in the synthesis of aromatic compounds.

Because the Calvin cycle and the PPP utilize many of the same enzymes in both pathways there are a number of regulatory mechanisms in place that control which pathway is operational at which time. The absence of light blocks the light reactions from taking place which causes a reduction in the pH of the stroma, reduced Mg^{2+} availability and the inactivation of the Calvin cycle enzymes: SBP, PRK, FBP and GAPDH. However, it has been identified that as many as 35 chloroplast enzymes that are involved in both pathways are regulated by thioredoxin (Balmer et al., 2003). In addition to the thioredoxin regulation of Calvin cycle enzymes, the key regulatory enzyme of the PPP, glucose-6-phosphate dehydrogenase (G6PDH) is also regulated by the same mechanism but is activate/de-activated in dark and light conditions respectively. The understanding of the regulatory mechanisms in both of these pathways is still being elucidated upon with multiple redox post-translational modifications thought to be involved in the regulatory process (Michelet et al., 2013).

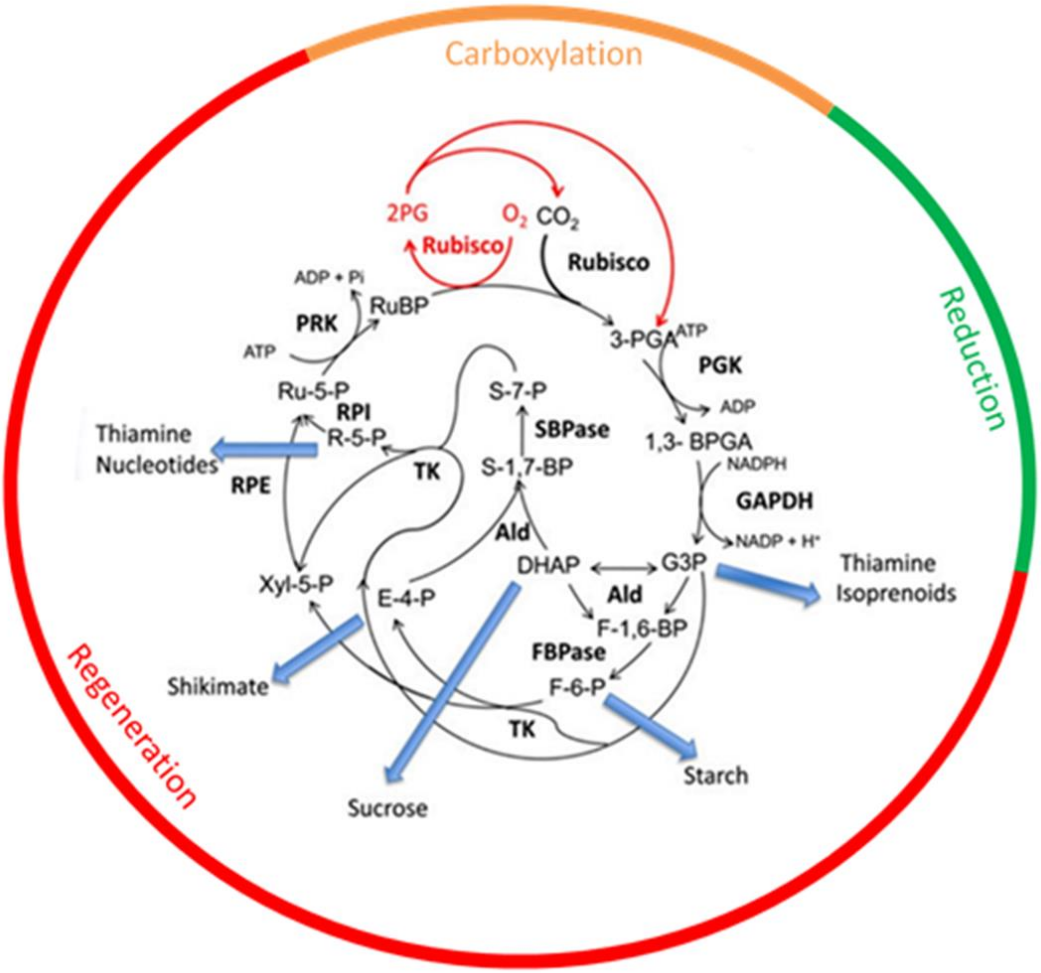

Figure 1.1- The C_3 cycle (Modified from Raines, 2011). The carboxylation reaction catalysed by RuBisCO that fixes CO_2 into the acceptor molecule RuBP, forming 3-phosphoglyceric acid (3-PGA).The reductive phase of the cycle follows with two reactions catalysed by 3-PGA kinase (PGK) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), producing glyceraldehyde-3-phosphate (G3P). G3P enters the regenerative phase catalysed by aldolase (ALD), and either FBPase or SBPase, producing fructose-6-Phosphate (F-6-P) and sedoheptulose-7-phosphate (S-7-P). F-6-P and S-7-P are utilized in reactions catalysed by transketolase (TK), R-5-P isomerase (RPI), and ribulose-5-P (Ru-5-P) epimerase (RPE), producing R-5-P. The final step converts Ru-5-p to RuBP, catalysed by phosphoribulokinase (PRK). The oxygenation reaction of rubisco fixes O_2 into ribulose-1,5-bisphosphate (RuBP), forming 3-Phosphoglyceric acid (PGA) and 2-phosphoglycolate (2PG), and the process of photorespiration (shown in red) releases CO_2 and PGA. The five export points from the pathway are shown with blue arrows.

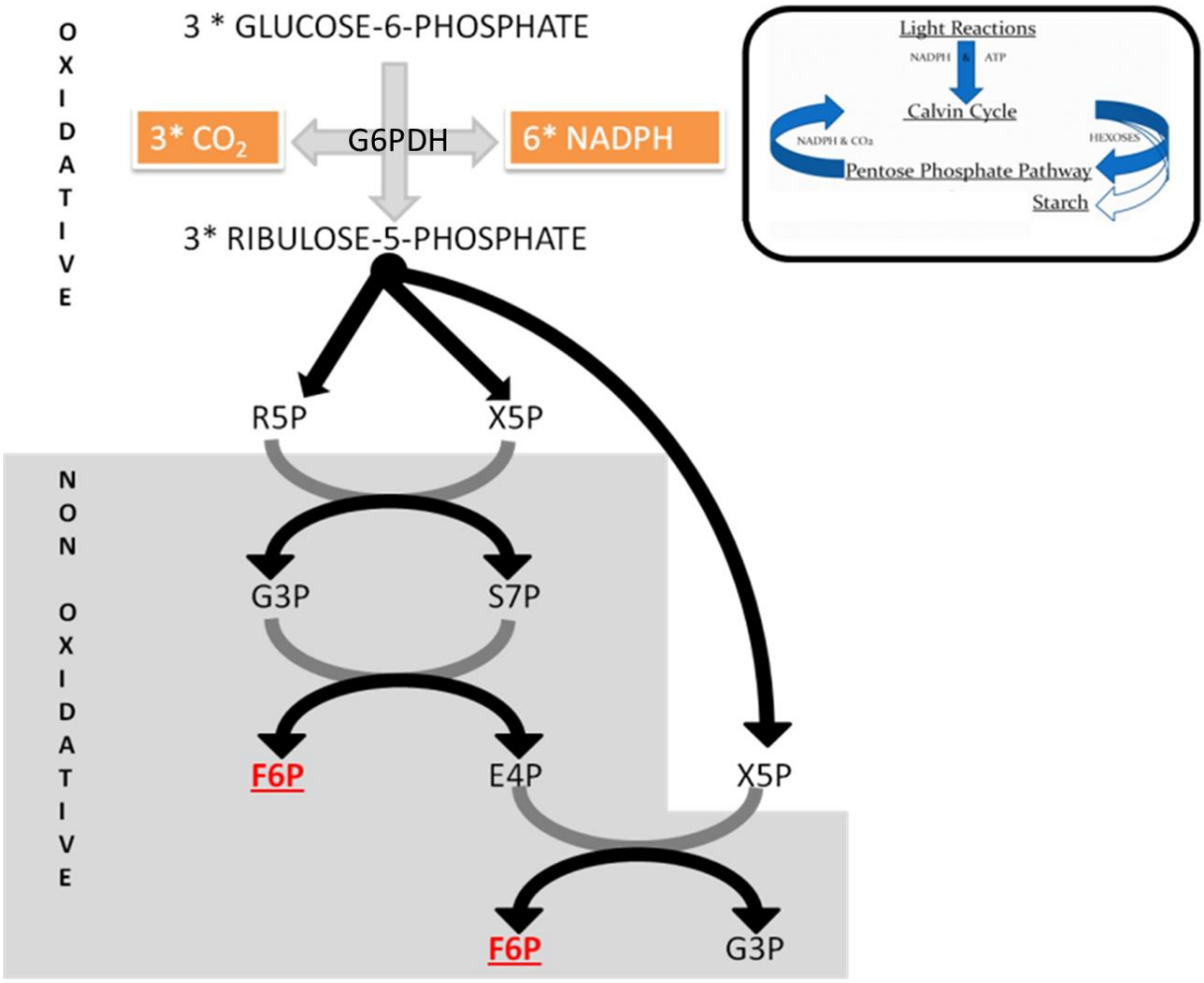

Figure 1.2- The Pentose Phosphate Pathway: three ribulose-5 phosphates are produced from three glucose-6-phosphates fed into the oxidative pentose phosphate pathway which is regulated by glucose-6-phosphate dehydrogenase (G6PDH). These are converted to one ribose-5-phosphate (R5P) and two xylulose-5-phosphate (Xu5P). Transketolase then acts to remove a two carbon fragment from XU5P and transfer it to R5P creating sedoheptulose-7-phosphate (S7P). Transaldolase then transfers a three carbon molecule from the S7P to the glyceraldehyde-3-phosphate (G3P) from the previous reaction creating fructose-6-phosphate (F6P). This leaves erythrose-4-phosphate which receives a two carbon fragment from the second transketolase reaction creating another F6P and one molecule of G3P. INSET: The light reactions produce NADPH and ATP that is then fed into the Calvin cycle where it fixes CO_2 to produce hexose sugars. These sugars can then enter into the pentose phosphate pathway or be used in starch production. The pentose phosphate pathway uses hexose sugars to produce pentoses and NADPH whilst releasing CO_2.

1.2 Identification of potential targets to improve photosynthesis.

The modification of individual enzymes within the Calvin cycle itself has produced results that have been shown to vary widely (Raines, 2003). In order to identify the relative importance in deferring the flow of carbon in the Calvin cycle, metabolic control analysis can be used. This is a powerful quantitative framework used in the understanding of relationships between biochemical steady-state networks as a whole and individual component reactions (Hofmeyr, 2001)

This approach assumes that no single enzyme has complete control over a pathway. Instead, all reactions are connected via common metabolites that, when combined, have a flux coefficient of one, indicating complete pathway control (Fell, 1992). It is important to note that any flux value for a single enzyme can vary depending on the individual conditions present during its analysis. The mathematical formula used to produce the flux coefficient value of a single enzyme is shown below:

C = flux co-efficient.

J = Original flux through the pathway.

δJ = change in flux.

E = original enzyme activity.

δE = change in enzyme activity.

$$C = \frac{\frac{\delta J}{J}}{\frac{\delta E}{E}}$$

The convergence of this information allows the understanding of plant metabolism at a systems level which permits the creation of detailed metabolic maps which when combined with computer prediction software allows the identification of potential alternative pathways for further investigation (Libourel and Shachar-Hill, 2008). The values themselves are most commonly identified through the use of antisense plants for the specific enzyme of interest.

1.3 Studies of Calvin cycle enzymes.

Aldolase (ALD) antisense studies demonstrated the effect that changes in the activity level of a single enzyme can have on the function of the Calvin cycle. Aldolase functions in the Calvin cycle by combining the two triose sugars D-glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP) into fructose 1,6-biphosphate. The reduction of ALD activity caused a decrease in photosynthetic rate, combined with reduced growth and starch synthesis, and a reduction in the regeneration capacity of RuBP (Haake et al., 1998).

The enzyme sedoheptulose-bisphosphatase (SBPase) functions in the process of RUBP regeneration through the dephosphorylation of sedoheptulose-1,7-biphosphate. In antisense studies it was shown that when levels of SBPase were reduced, a negative effect was seen on the plant, in that the reduced levels of the protein lead to a decrease in RuBP regeneration (Harrison et al., 2001), which was found to reduce growth, delay the onset of flowering (Lawson et al., 2006) and to alter photosynthetic operating efficiency in guard cells (Lawson et al., 2008).

Because of the finding that reductions in SBPase activity had a negative effect on growth it was thought that an increase in SBPase levels may increase plant growth. Plants with increased SBPase activity showed an increase in photosynthetic rates, as well as an increase in biomass of up to 30%. In addition, they also provided evidence that it is not necessary to bypass the regulatory redox control of SBPase in the stroma to improve carbon fixation (Lefebvre et al., 2005). An increase in SBPase activity has also been found to increase high temperature tolerance (Feng et al., 2007) and increase growth under salt stress conditions (Feng et al., 2009).

Studies of TK antisense tobacco plants found that when levels of TK activity were reduced between 20% – 40 % both RuBP regeneration and photosynthesis were limited. In addition, a reduction in TK activity also caused a decrease in sugars, whereas starch remained high until photosynthesis was strongly inhibited. A further decrease in TK activity of up to 50 % had a direct effect on phenylpropanoid metabolism, whilst decreases of > 50% caused loss of chlorophyll and carotene on the midrib (Henkes et al., 2001). These findings raise the possibility that an increase in TK activity may have a beneficial effect, as had been observed in the SBPase sense mutants.

Tobacco plants (*Nicotiana tabacum L. cv. Samsun*) with increased total TK protein levels of between 2 and 2.5 times that found in the WT produced chlorotic phenotypes that were able to be rescued by germinating them in a thiamine enriched media (Khozaei, 2010). The phenotype of these plants was similar to that found by McHale et al., 1988 where disruption of chlorophyll and carotenoid synthesis in *Nicotiana sylvestris* also caused a chlorotic phenotype. In the latter case the phenotype was attributed to insufficient levels of the TPP co-factor for pyruvate dehydrogenase to produce acetyl-coenzyme- A. However, they are many other examples where a similar chlorotic phenotype has been seen. These include inhibition of the DXS enzyme (Matsue et al., 2010), inhibition of triose phosphate isomerase (Chen and Thelen, 2010) and reduced transport of phosphoenolpyruvate to plastids (Prabhakar et al., 2010).

It was found that only thiamine or TPP supplementation at the point of germination was able to complement the growth and chlorotic phenotypes. This correlates with the finding that thiamine deficiency may lead to premature termination of meristems in

seedlings leading to reduced growth capacity (Woodward et al., 2010), indicating the importance of thiamine in seedling establishment.

1.4 The mechanism of transketolase.

TK functions by catalysing the transfer of a two carbon fragment from a ketose sugar such as xylulose-5-phosphate (Xu5P) to an aldose sugar like erythrose-4-phosphate. This reaction releases glyceraldehyde-3-phosphate (G3P) from the initial Xu5P and forms fructose-6-phosphate (F6P) when the removed fragment is attached to E4P. F6P is involved in starch synthesis; E4P is a precursor for the shikimate pathway leading to phenylpropanoid metabolism whilst R5P is involved in nucleotide and thiamine biosynthesis.

The active sites of TK, that is those sites which interact with the substrates and co-factors, are found at the interface between two identical TK subunits. These subunits are individually composed of three domains which are identified as the: pyrophosphate (P-P), pyrimidine (Pyr) and C-terminal. All TPP dependent enzymes possess both the PP and Pyr domains (Widmann et al., 2010). To function, TK requires the co-factor thiamine pyro-phosphate (TPP) that binds to both the P-P and Pyr domains. TPP binds in the cleft formed by the PYR and PP domain with each domain binding a different part of the TPP molecule. The PYR domain, binds the aminopyrimidine ring of TPP whilst the PP domain binds the diphosphate residue. These subunits have been shown to operate independently, with one subunit cleaving the ketose whilst the other subunit completes the joining of the 2 carbon fragment to the aldose (Sevostyanova et al., 2009). In addition to the binding of TPP, TK also requires a second co-factor in the form of the cations Ca^{2+}, Mg^{2+} or Mn^{2+}. In plants little is known

about the regulation of TK though it has been shown to be involved in the rehydration process of resurrection plants (Bernacchia et al., 1995).

In yeast the availability of the different cation species such as Mg^{2+} has been linked with a change in TK substrate affinity, as a switch from Mg^{2+} to Ca^{2+} causes one of the subunits to significantly decrease its affinity for the substrate Xu5P (Sevostyanova et al., 2008). In addition to cation substitution impacting upon substrate affinity, the substrate itself has been shown in yeast to influence the affinity of TK for its TPP co-factor. High levels of Xu5P cause the non- equivalent binding of TPP in TK's active centres. Whilst prevalence of F6P increases the inactivation rate of TK (Kochetov and Sevostyanova, 2010). This indicates that TK is controlled by a number of complex biochemical interactions attributed to both the type and quantity of both co-factors and substrates present. The TPP co-factor is also required by a number of other enzymes that are involved in the formation of acetyl-CoA, the citric acid cycle (TCA), the non-mevalonate pathway (MEP) and branched chain amino acid metabolism (BCAA) (Figure.1.3)

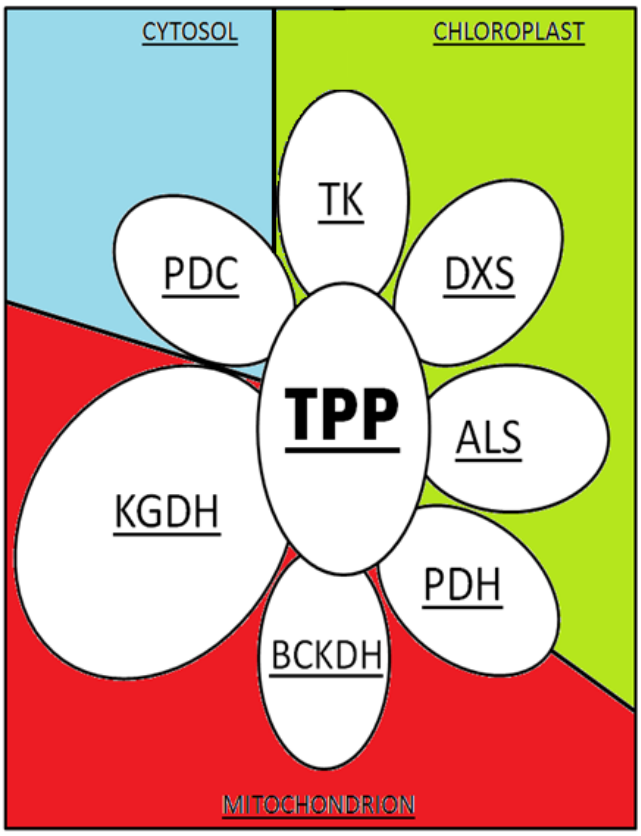

Figure 1.3- Enzymes dependent on the co-factor TPP and their cellular location. (modified from Kozik et al.,2012). thiamine pyrophosphate (TPP); pyruvate decarboxylase (PDC); pyruvate dehydrogenase (PDH); a-ketoglutarate dehydrogenase (KGDH) (key regulatory enzyme complex of TCA cycle); (BCKDH) branched-chain a-ketoacid dehydrogenase (KDH); transketolase (TK); 1-deoxy-D-xylulose-5-phosphate synthase (DXS); acetohydroxyacid synthase (ALS)

1.5 Thiamine biosynthesis.

The biosynthesis of thiamine takes place in the chloroplast (Belanger et al., 1995) where the condensation of the pyrimidine heterocycle (HMP-PP) and the thiazole heterocycle (HET-P) form thiamine mono phosphate (TMP) (Figure.1.4). HMP-PP is formed from aminoimidazole ribonucleotide (AIR) which utilizes R5P in the creation of its purine ring structure whilst the HET-P moiety in eukaryotes is formed by the initial condensation of NAD+ and glycine (Begley et al., 2012) whilst in bacteria it is produced by condensation of pyruvate and G3P by 1-deoxy-D-xylulose-5-phosphate synthase (DXS) to form to 1-deoxy-D-xylulose 5-phosphate (DXP) (Jurgenson et al., 2009). However, there is much ambiguity around this area as there is also evidence to show that plants are able to produce the thiazole moiety from utilizing DOXP as in bacteria (Julliard and Douce, 1991a).

Based on this knowledge, the DXS enzyme occupies a position where its product 1-deoxy-d-xylulose-5-phosphate (DOXP) is essential for both the production of the thiazole molecule utilized in thiamine biosynthesis as well as the MEP pathway which is employed in the synthesis of isoprenoids, chlorophylls and plant hormones. TPP, the phosphorylated form of thiamine, is therefore essential for creation of the DOXP intermediate which allows the end products to be created in both of these pathways.

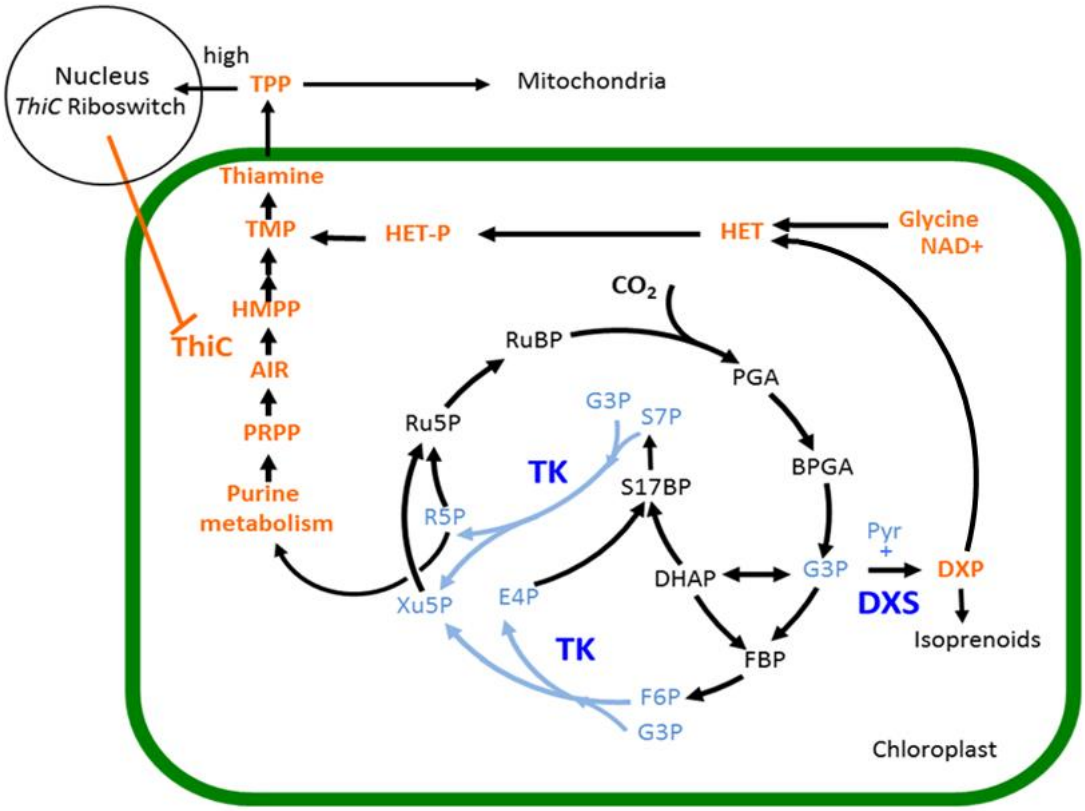

Figure 1.4-The C_3 cycle showing relationships to the isoprenoid and thiamine biosynthesis pathways. Thiamine biosynthesis (red typeface) is formed from two moieties, hydroxymethylpyrimidine pyrophosphate (HMP-P) and hydroxyethylthiazole phosphate (HET-P) that are biosynthesised from compounds arising from the C_3 cycle, ribose 5-phosphate (R5P) and glyceraldehyde 3-phosphate (G3P). These two compounds are respectively a product and substrate of the reactions carried out by transketolase (TK). Glycine and NAD+ can also be used to produce hydroxyethylthiazole (HET). Thiamine monophosphate (TMP) is generated from the condensation of HET-P and HMPP. TMP is dephosphorylated to thiamine which is transported to the cytosol where it undergoes phosphorylation to thiamine pyrophosphate (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.

1.6 The biosynthesis and regulation of the thiazole moiety of thiamine.

DXS is a TPP dependent enzyme that is important not only for the production of isoprenoids but also the biosynthesis of the thiazole moiety of thiamine (Julliard and Douce, 1991b). The DXS enzyme has been found to be highly regulated by a number of factors including both the transcript and protein levels of the enzyme (Guevara-Garcia et al., 2005), light (Loivamaki et al., 2007), as well as the MEP pathway products where both isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) were shown to compete with TPP for binding with the enzyme (Banerjee et al., 2013). Supplementation with 1-deoxy-xylulose-5-phosphate (DOXP) has been shown to bypass DXS (Julliard and Douce, 1991a). For reviews see: (Cordoba et al., 2009; Banerjee et al., 2013; Banerjee and Sharkey, 2014).

A relationship has been found between TK and DXS protein levels. In tobacco plants with increased TK protein levels increases in the level of the DXS protein were also measured (Khozaei, 2010). The enzyme 1-deoxy-d-xylulose-5-phosphate reductoisomerase (DXR) is the second enzyme in the MEP pathway and this controls flux to the MEP pathway away from thiazole biosynthesis. DXR was found to be up regulated through the chloroplast to chromoplast transition (Kahlau and Bock, 2008), whilst suppression of DXR caused an albino dwarf phenotype that had reductions in both gibberellic acid (GAs) and abscisic acid (ABA); the synthesis of these is co-ordinately regulated (Xing et al., 2010).The enzyme Thi1 converts DOXP to (4-methyl-5-(β-hydroxyethyl)thiazole) HET. Studies of a Thi1 homologue in yeast identified that enzyme produced can only perform one reaction which makes the thiazole moiety very costly to produce (Chatterjee et al., 2011). In algae, Thi1 has been shown to contain a riboswitch mechanism allowing regulation of the production of the thiazole moiety of thiamine (Moulin et al., 2013).

1.7 The biosynthesis and regulation of the pyrimidine moiety of thiamine.

The pyrimidine heterocycle of thiamine is derived from 5-amino-imidazole-ribonucleotide (AIR) which is converted to hydroxymethyl pyrimidine phosphate (HMP-P) by the enzyme HMP-P synthase (ThiC) which requires S-adenosyl-methionine (S-AM) and reduced nicotinamide adenine dinucleotide (NADH). Biosynthesis in both eukaryotes and prokaryotes is thought to share high similarity. The ThiC gene from A. thaliana was found to complement an *Escherichia* coli ThiC mutant (Kong et al., 2008). The next stage in the pathway proceeds with the phosphorylation of HMP-P by the dual function enzyme hydroxymethyl pyrimidine kinase (TH1) which also combines both the thiazole and pyrimidine moieties to form thiamine monophosphate in the chloroplast (Ajjawi et al., 2007).

The biosynthesis of the pyrimidine moiety of the thiamine molecule is controlled by a regulatory mRNA element known as a riboswitch which is located within the 3' region of the ThiC gene (Raschke et al., 2007; Wachter et al., 2007; Kong et al., 2008). Riboswitches are found in all domains of life and function through the binding of a preferential ligand to an mRNA molecule. The binding of the ligand causes a conformational change in the mRNA structure which alters gene expression via the adjacent open reading frame (ORF) which can affect either the splicing or the translational properties of mature mRNA which is determined by the quantity of substrate present (Vitreschak et al., 2004).

Riboswitches can be divided into an aptamer region, which binds to a specific target molecule, and an expression region, which undergoes a structural change in response to the binding of the target molecule to the aptamer site. The aptamer region is used to classify riboswitches depending on the affinity of their aptamer site to the specific

substrate. Riboswitch substrates include amino acids, carbohydrates, and co-enzymes such as TPP (Bastet et al., 2011). In addition to regulation through the binding of small metabolites, riboswitches can also be regulated via temperature (Henkin, 2008) and pH (Bingham et al., 1990). Riboswitches are essential for the efficient regulation of resources for the specific conditions and are thought to represent one of the oldest known gene regulatory systems (Vitreschak et al., 2004).

One of the most common riboswitches is the TPP riboswitch and is the only riboswitch that is found in plants, archea and microorganisms (Miranda-Rios, 2007). Though its mode of operation differs slightly through its sequence and splicing variations, all TPP riboswitches achieve the same outcome of controlling the synthesis of thiamine through variation in the splicing of the nascent mRNA and the positioning of the TPP riboswitch in the mRNA strand (Henkin, 2008). The operation of the TPP riboswitch in plants is dependent on the alternate splicing of nascent mRNA dependent on the quantity of TPP present. The level of cellular TPP that flips the TPP riboswitch has been shown to be between 100 nM and 1 μM (Bocobza et al., 2007). Levels of Mg^{2+} have been shown to vary this reaction in bacteria (Bocobza et al., 2007) which suggests that riboswitch activity is able to undergo gradual changes to suit the conditions, rather than simply be switched on or off. More recently, regulation of the ThiC riboswitch in algae which regulates the pyrimidine moiety has been shown to be controlled by HMP-PP as well as by TPP (Moulin et al., 2013). The circadian clock also has a role in ThiC regulation via its promoter (Bocobza et al., 2013)

The TPP riboswitch in plants functions through the binding of TPP to the unprocessed mRNA strand which causes specific folding and exposure of the 3' and 5' splice sites leading to a long unstable mRNA transcript which is ultimately degraded (Figure.1.5). This prevents ThiC translation and hence formation of the pyrimidine moiety of

thiamine. In the presence of low levels of TPP this folding does not occur which allows the translation of a short and stable mRNA molecule that permits ThiC translation (Bocobza and Aharoni, 2008).

Before TMP can be phosphorylated to TPP it is first dephosphorylated to thiamine, and transported across the chloroplast membrane into the cytosol where the enzyme thiamine pyro-phosphokinase (TPK), which has both kinase and phosphatase properties, can regulate the proportion of the thiamine variants available (Rapala-Kozik et al., 2009). Thiamine has yet to be connected with any specific role but TMP is said to constitute a thiamine resource or transitional stage for TPP synthesis (Rapala-Kozik et al., 2012).

1.8 Thiamine transport and compartmentation.

In addition to the riboswitch regulation of thiamine metabolism it is also subject to further control through compartmentation. TPP in its active form is the co-factor for a number of enzymes that reside in different cellular compartments (Figure.1.6). In plants only mitochondrial TPP transporters have been identified (Frelin et al., 2012). Thiamine has been identified as being synthesized in chloroplasts (Belanger et al., 1995) . Therefore a transport mechanism must exist to permit export of thiamine to the cytosol where it is phosphorylated to TPP which provides a further regulatory layer to thiamine metabolism (Moulin, 2013)

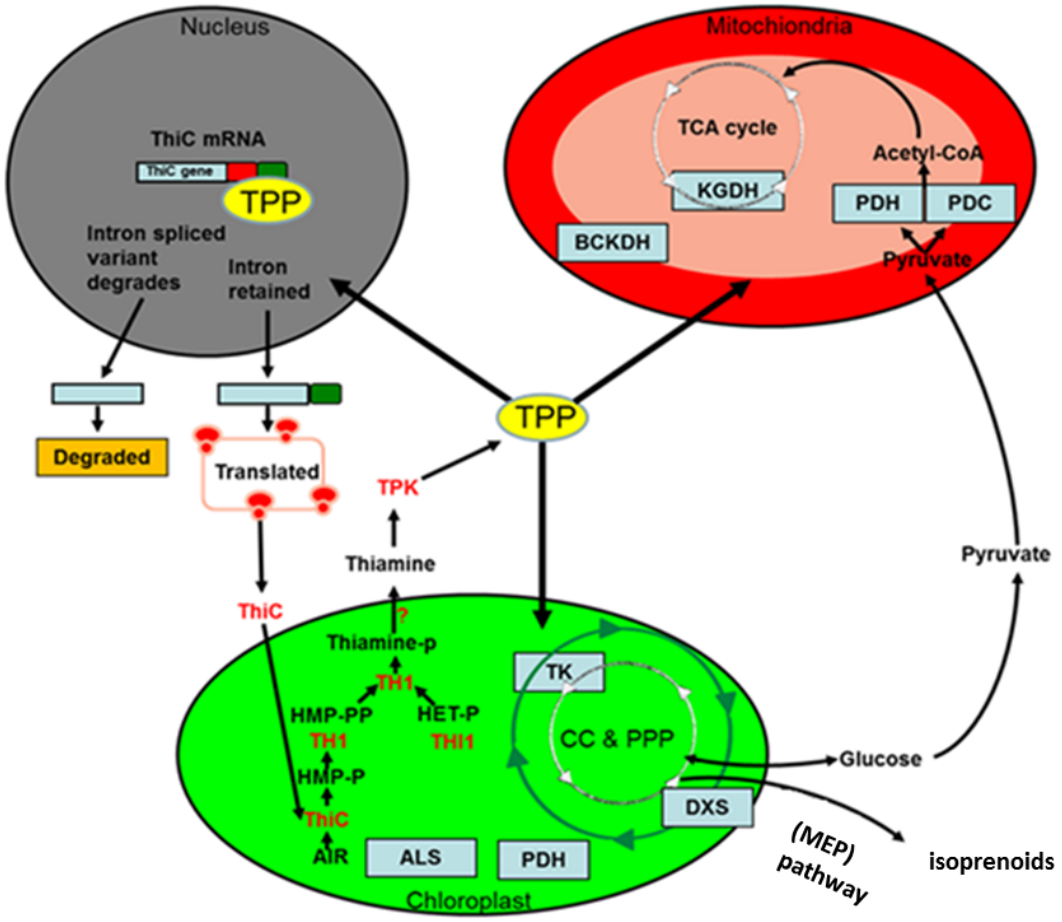

Figure 1.5-Thiamine metabolism compartmentation and reactions (redrawn and modified from Bocobza et al., (2013) Thiamine mono-phosphate (TMP) is produced in the chloroplast from HMP-PP and HET-P. It is then dephosphorylated and exported into the cytosol where it is phosphorylated by the enzyme thiamine phosphokinase to TPP. TPP can then re-enter the chloroplast to act as a co-factor for transketolase (TK), pyruvate dehydrogenase (PDH) and deoxy-xylulose synthase (DXS) and acetolactate synthase (ALS). TPP from the cytosol can also enter the mitochondria as a co-factor for pyruvate dehydrogenase (PDH), pyruvate decarboxylase (PDC), ketoglutarate dehydrogenase (KGDH) and branched chain ketoacid dehydrogenase (BCKDH). It also crosses the nuclear membrane to regulate the nascent mRNA of ThiC.

1.9 Thiamine storage during seed development.

Thiamine availability is essential during seed germination to permit the mobilisation of seed reserves by providing sufficient levels of TPP for the TPP dependent enzymes to function until the plant is able to synthesize its own supply of thiamine. An increase in thiamine availability has been found to enhance the germination process (Neumann et al., 1996).

The biosynthesis of thiamine as indicated by the presence of ThiC mRNA transcripts has been identified as early 5 days after germination (Raschke et al., 2007) whilst studies that measured actual thiamine levels found biosynthesis to begin at 12 days (Johnson and Goodwin, 1965). Therefore, it is essential that the seedling has sufficient levels of stored thiamine to progress through the early stages of growth until the plant is able to synthesize its own supply of thiamine.

A thiamine storage facility is provided by thiamine binding proteins (TBPs) which are located in the aleuronic layer of the embryo and act as storage molecules during seed development (Mitsunaga et al., 1986). Thiamine levels have been shown to be at levels tenfold higher in the seed than the leaves (Shimamoto and Nelson, 1981). The seed bound thiamine is retained during seed dormancy. Once the dormancy stage is completed, thiamine is released and converted into its active TPP form where it contributes to endosperm growth and seed rupture (Ampo et al., 2007).

TBP's have been studied in a number of plants including *Zea* Mays*, Vicia* faba *and Pisum* sativum (Golda et al., 2004). Studies in *Triticum* aestivum showed that TBPs were found in the aleuronic layer during seed maturation and consisted of two subunits both of which had high levels of the amino acid glutamine (Watanabe et al., 2004). The origin of thiamine that becomes bound to TBP's is not fully understood.

The gene transcripts for enzymes involved in the synthesis of the thiazole and pyrimidine moieties of thiamine have been shown to have a divergent expression pattern in seeds and leaves. This may indicate that thiamine is not transported to thiamine binding proteins as a whole but rather through its constituent parts (Guan et al., 2014).

TBP denaturation and therefore thiamine release occurs below pH 5, whilst optimal thiamine binding to TBPs during seed development occurs at pH 7.5 (Kozik, 1995). The breakdown of TBPs and therefore the release of thiamine have been shown to vary across plant species. *A. thaliana* has a thiamine breakdown rate of 5 % per day (Raschke et al., 2007) whilst the levels in *Triticum* species are estimated to be 2 % (Golda et al., 2004) and 13 % in *Sesanum* Indicum (Watanabe et al., 2003).

1.10 The role of thiamine variants in the plant stress response.

Thiamine and TPP levels have been shown to be increased when the plant is exposed to osmotic stress (Rapala-Kozik et al., 2012), temperature change (Cui et al., 2005) or bacterial/viral attack (Ahn et al., 2005).

The total amount of the different thiamine derivatives (thiamine, TMP and TPP) under stress conditions was found to increase by 380 %, compared to that measured in unstressed controls (Rapala-Kozik et al., 2012). In addition to an increase in the expression of the thiamine biosynthesis genes: Thi1, ThiC, TH1 and TPK, the expression levels of other stress related genes were also found to be increased. These included pathogen related proteins (PR-1) which provide fungal resistance (Ahn et al., 2005) and include the stress signalling hormones: abscisic acid (ABA) and

jasmonates as well as the TPP dependent enzymes: TK, KGDH, DXS and PDH (Rapala-Kozik et al., 2012). Thiamine deficiency in animals causes a reduction of between 10.5 and 24 % in tricarboxylic acid cycle enzyme activity by both TPP dependent and non TPP dependent enzymes (Bubber et al., 2004). A similar study in plants is not currently available.

1.10 The role of thiamine pyrophosphate (TPP).

Thiamine pyrophosphate (TPP) is known as the main biologically active thiamine variant that acts as a co-factor for both TK and DXS enzymes as well as a number of other enzymes which are found in both mitochondrial and plastid complexes (Rapala-Kozik et al., 2012). These TPP dependent enzymes include pyruvate dehydrogenase (PDH) which produces acetyl-coenzyme A (Acetyl CO-A) from pyruvate in both the mitochondria and chloroplast. However, the Acetyl CO-A produced in both organelles is utilized differently. In the mitochondria it links glycolysis to the TCA cycle whilst in plastids it is used in the synthesis of fatty acids. In addition, the enzyme pyruvate decarboxylase (PDC) functions to break down pyruvate to acetaldehyde and was shown to be vital for energy production under anoxia in Arabidopsis (Gass et al., 2005). Whilst a second mitochondrial TPP dependent enzyme, ketoglutarate dehydrogenase (KGDH) converts α-ketoglutarate, coenzyme A and NAD+ to succinyl-CoA, whilst releasing NADH and CO_2 as part of the process. KGDH is a highly regulated enzyme and is the primary control site of metabolic flux through the Krebs cycle (Hansford 1980).

The branched chain amino acids valine, leucine and isoleucine which are only produced in plants are dependent on the TPP dependent enzyme acetolactate

synthase (ALS). This enzyme catalyses the conversion of two molecules of pyruvate into 2-acetolactate which is the first reaction in a three step pathway used to produce the three amino acids (Binder, 2010). A second TPP dependent enzyme, branched chain ketoacid dehydrogenase (BCKDH), is involved in the breakdown of the branched chain amino acids and has been identified as having a significant role in amino acid metabolism in Arabidopsis (Peng et al., 2015).

1.11 Conclusion.

The modification of individual enzymes, such as TK, has shown how complex the system of regulation is within both the Calvin cycle and the many interlinked pathways that are themselves dependent on the availability of specific substrates, as well as the co-factors required for enzymatic function. Co-factors such as TPP have shown that there are yet further layers of regulation which include riboswitches and mRNA processing which can be altered depending on the physiological requirements of the plant.

The discovery that over expression of TK itself has a direct effect on thiamine metabolism suggests that a further unknown regulatory pathway may be involved; if this pathway is able to be identified then it may be possible to produce transgenic plants which may produce both a higher yield and allow a greater resistance to stress which is the primary cause of crop loss (Boyer, 1982). To achieve this the plant would also require increased thiamine levels; this factor is already being investigated by plant scientists (Goyer and Haynes, 2011), whilst riboswitch (Costa, 2009) and TK regulation and manipulation (Nobeli et al., 2009) have both generated significant interest both in and outside of the plant scientific community.

With the knowledge gained from these studies it is possible that crops with increased thiamine content may be produced, which would be beneficial to health, but additionally that this characteristic may also enhance agricultural productivity.

1.12 Aims of this project.

A previous study (Khozaei, 2010) showed that an increase in the level of non-native plastid TK in *Nicotiana tabacum* had a detrimental effect on plant growth and development and also causes a chlorotic phenotype. Both of these phenotypes were able to be rescued through the availability of an external thiamine source during the germination period. Therefore, the aim of this project was to investigate the relationship between TK expression, the Calvin cycle and thiamine metabolism. To do this I will be using tobacco plants (*Nicotiana tabacum* L.cv. Samsun) that were shown to have increased TK activity of ~2 fold higher than that of WT plants.

To achieve this I first identified the growth and photosynthetic characteristics of these plants in relation to varying thiamine supplementation regimes at different stages of development. Then, using the knowledge gained from this investigation I investigated the effect of increased TK activity on thiamine metabolism.

CHAPTER 2

MATERIALS AND METHODS

Plant growth and analysis

2.1 Plants used in this study.

The plants used in this study contained a full-length plastid transketolase cDNA (Atg360750) that was cloned into the pMog 22 vector containing the cauliflower mosaic virus (CaMV) 35S promotor and nopaline synthase (NOS) terminator sequence (Lefebvre, 2005). The recombinant plasmid was introduced into tobacco (*Nicotiana tabacum* L. 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 in order to generate lines of third generation (T3).

2.2 Plant material and growth analysis.

The seeds of *Nicotiana tabacum* L.cv. Samsun of both WT and mutant lines (Tkox -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 minutes and rinsed and re-suspended in sterile water and stored at 4^{o} C for 24 h. Seeds were germinated on 0.8% (w/v) agar containing 0.44% Murashige and Skoog medium (MS) 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^{o} C, 16 h light/8 h dark, light levels of 200 µmol $m^{-2}s^{-1}$. At ~16 days post germination, seedlings were transferred to soil, placed in the greenhouse and irrigated with Hoaglands solution.

2.3 Growth analysis of younger plants.

The growth of younger plants was measured by photographing the plants from above and then by measuring the rosette diameter using Image-J software, version 1.43u (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

2.4 Root Growth analysis.

Primary root length was measured by positioning the plates vertically in the growth chamber. This was photographed and the length of the primary root was measured using Image-J software, version 1.43u (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

2.5 Growth methods dependent on developmental stage.

WT and TKox lines were germinated in seed trays filled with compost. The trays were placed in a growth cabinet for 16 h light/8 h dark at light levels of 150 µmol m^{-2} s^{-1} and a temperature of 22^{o} C. Samples were taken at the midpoint of the photoperiod when they reached the growth stages outlined previous.

Plant material was harvested into the following tissue types: cotyledons, 1st true leaf, and 2nd true leaf at each of the developmental stages. All material was immediately snap frozen in liquid nitrogen and stored at -80^{o} C for later analysis.

2.6 Growth and sampling conditions for developmental stage experiment.

Seeds of WT and the three TKox lines were grown on MS plates with each line having three standard plates and three supplemented with thiamine (50 Mg L^{-1}) supplemented plates. The plates were placed in a growth cabinet for 16 h light/8 h dark at light levels of 150 µmol $m^{-2}s^{-1}$ at a temperature of 22^{o} C. At 12 days post germination 20 plants from each WT and Tkox line were transferred to potting trays containing Levington F2 compost (Levington, Ipswich, Suffolk, UK). At this stage the thiamine supplementation was changed so that 25% of those plants that had not received thiamine supplementation in their germination media now received thiamine (50 Mg L^{-1}) via irrigation. A further 25% of plants that did receive thiamine previously had their thiamine supplementation withdrawn. The remainder of the plants continued as per their previous regime. At 17 days post germination the plants were transferred to larger pots and placed in long day conditions in the greenhouse. The thiamine supplementation regime continued as previously described throughout the lifecycle of the plants.

2.7 Leaf area and dry weight analysis.

At thirty three days post germination all leaves including petioles were removed from the plants, photographed and then placed in the drying oven at 70^{o} C until it was sufficiently dried (~4 days). The leaf area of each plant was calculated using Image-J software, version 1.43u (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2014).

2.8 Gas exchange analysis.

Aci curve

The response of assimilation rate of CO_2 (A) to intercellular CO_2 concentration (Ci) was determined at a saturating light level of (1200 µmol photons m^{-2} s^{-1}) with a leaf temperature of 25^{o} C and a VPD 1 (+/- 0.2) KPa using an infrared gas analyzer (IRGA) (CIRAS-1;PP-Systems, Hitchin, Herts. UK). Prior to use, the IRGA was calibrated against a known CO_2 standard (Linde Gas Ltd, Stratford, London, UK).

Photosynthetic carbon fixation rates were measured at a range of CO_2 concentrations from 50 to 1300 µmol mol^{-1}. Measurements were started at the ambient CO_2 concentration (Ca) of 400 µmol mol^{-1} and then levels were decreased to 250, 100 and 50 µmol m^{-1}. Levels were returned to 400 µmol mol^{-1} to confirm the original rate could be regained using the same steps. Following this, the Ci was increased by incremental steps to 550, 700, 900, 1100, 1300 µmol mol^{-1}. The data produced was used to produce an ACi curve.

2.9 Molecular biology techniques

2.10 Primer design.

All primers used in this study were based on transcript sequences, from the NCBI database. The online tools used to design primers were:

- https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp
- http://biotools.umassmed.edu/bioapps/primer3_www.cg

All qPCR primers were designed to have an annealing temperature of 60^{o} C with an optimal length of < 100 base pairs.

2.11 Polymerase chain reaction (PCR).

PCR reactions were performed using Dreamtaq (Thermo Scientific) in a 10 µl reaction. The reaction mix consisted of: 6.6 µl H_2O, 0.2 µl dNTPs (10 mM stock), 0.5 µl forward and reverse primers (10 pmol µl^{-1} stock), 0.2 µl Dreamtaq polymerase and 1 µl of DNA/CDNA.

2.12 Quantative polymerase chain reaction (qPCR).

The qPCR reactions were performed using SensiFAST SYBR kit (Bioline). The 15ul mix consisted of: 7.5 µl Bioline sybr mix, 0.75 µl forward and reverse primers (10 pmol µl^{-1} stock) 6 µl CDNA (2.1 ng ul^{-1}). Reactions were run in triplicate using the CFX96 Touch system (Biorad). No template controls (NTC) and no reverse transcription (NRT) controls were used to assess the integrity of the results. Primer efficiency was determined by diluting the CDNA sample and producing a standard curve as follows: 1, 1:10: 1:100 1:1000 1:10000. Only primers that had an efficiency value between 90 and 110% were used.

The qPCR cycling conditions were: initial denaturation of 95^{o} C for 2 mins to activate the Taq polymerase, 35 cycles at 95^{o} C for 5 s for denaturation, annealing at 60^{o} C for 10 s and extension at 72^{o} C for 5 s. The amplification was followed by a melting curve analysis which ranged from 65^{o} C to 90^{o} C with a temp increase of 0.2^{o} C every 10 s. Baseline and threshold cycles (Cq values) and primer efficiency were determined using Bio-Rad CFX manager software (v3.1).

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 is deemed not to interfere with the final result (Exposito-Rodriguez et al., 2008).

2.13 Agarose gel electrophoresis.

Nucleic acids were separated using agarose gels at either 1% or 2% agarose concentration. TAE buffer was used for gel preparation and consisted of the following final concentrations: 40mM TRIS, 20mM acetic acid, and 1mM EDTA. Nucleic acids were stained using Safeview (Nbs biologicals ltd.) and viewed under blue light.

2.14 RNA extraction.

Plant RNA was isolated using Tri-reagent (Sigma). Approximately 100 Mg of plant material was first ground into a fine powder and then transferred into a 2 ml Eppendorf tube and kept on dry ice. 1 ml of Tri-reagent was added and the mixture was vortexed for 20 s then left at RT for 5 mins and finally centrifuged at 4^{o}C for 10 mins at 12000g. 500 µl of the aqueous phase was removed and transferred to a new 1.5 ml Eppendorf. 200 µl of chloroform was added, mixed by inversion and left at RT for 2 mins. The solution was centrifuged at 4^{o} C for 10 mins at 12000g. The aqueous phase of ~ 300 µl was placed in a new Eppendorf and mixed with 300 µl of chilled isopropanol, mixed by inversion and incubated at RT for 1 minute and centrifuged at 4^{o} C for 10 mins at

12000g. The RNA pellet was washed with 1 ml 75 % chilled ethanol and centrifuged at 4^{o} C for 5 mins at 12000g. The ethanol was poured away and the pellet air dried for 5 mins. The pellet was resuspended in 20 µl of sterile water. The purity and concentration of RNA was conducted using a Nanodrop system (Thermo-scientific). To assess RNA quality, 400 ng of RNA was run on a 2 % TAE agarose gel to determine clear ribosomal bands which are indicative of non-degraded RNA.

2.15 cDNA synthesis.

Before cDNA synthesis, DNA contamination was removed using a DNAse1 kit (Invitrogen) following the manufacturer's instructions. The CDNA synthesis reaction was achieved by adding 1 µl of oligo dT's (an equal mix of deoxy-thymine nucleotides of between 15 and 18 nucleotides in length), 1 µl of DNTP mix and 1 µg of purified RNA. The solution was made up to 12 µl with RNase free water, incubated for 10 mins at 65^{o} C and kept on ice for 7 mins. To the tube was added 4 µl of 5 x reaction buffer, 1 µl of DTT and 1 µl of the reverse transcription enzyme. This mixture was centrifuged briefly and then incubated for 60 mins at 42^{o} C and 5 mins at 70^{o} C.

2.16 RNA sequencing (RNA-seq) comparison of WT and TKox -1 RNA.

RNA extraction and quality check

Three biological reps of each line were sampled from plants described in section 2.1. RNA extraction was conducted independently for each of the three samples used for both WT and Tkox -1 lines. To assess purity and sufficient quality for RNA seq analysis, each RNA extract was tested on the Agilent 2100 Bioanalyzer system where

28S/18S values were shown to be between 1.5 and 1.9. These values indicate sufficient RNA quality based on specifications from Genome Enterprise Limited (GEL) at The Genome Analysis Centre (Norwich, UK). Two pooled samples (3*WT and 3*Tkox -1) were sent for analysis with each comprising of equal amounts (1.66 µg per sample) of total RNA (5 µg).

Sequencing

RNA samples were sequenced by Genome Enterprise Limited (GEL) at The Genome Analysis Centre (Norwich, UK) using Illumina HiSeq 2000 (Illumina, San Diego USA); using 51bp paired end reads, insert length ~200bp. The data was de-multiplexed and quality checked using FastQC (Andrews, 2010).

2.17 Gene ontology analysis.

For gene ontology (GO) analysis TopGO was used from Bioconductor in R (Gentleman et al., 2004). This was done for all differentially expressed genes, split between over and under-expressed genes identified by EdgeR (Robinson et al., 2010), for each pair wise comparison. Fishers test was implemented in TopGO to identify enriched GO terms per comparison. From this, all GO terms with a $P < 0.01$ were selected.

2.18 Analysis of processed RNA-seq data.

The processed transcript levels of independent genes were analysed by splitting the dataset into three groups:

1: All transcripts that differed from WT

2: Transcripts that were up-regulated from WT.

3. Transcripts that were down-regulated from WT.

The resulting gene ontology (GO) terms were analysed using the freely available online tools:

- DAVID (Database for Annotation, Visualization and Integrated Discovery) http://david.abcc.ncifcrf.gov/

- agriGO (gene ontology analysis toolkit) http://bioinfo.cau.edu.cn/agriGO/

- STRING Search Tool for the Retrieval of Interacting Genes/Proteins http://string-db.org/Biochemistry techniques

2.19 Protein extraction.

Plant tissue was snap frozen in liquid nitrogen and then ground to a fine powder. 100 Mg of this powder was added to 1 ml of 50 mM 4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) (pH 8.2) buffer containing: 5 mM MgCl2, 1 mM, ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 0.1% Triton X, 2 mM benzamidine, 2 mM aminocaproic acid, 0.5 mM phenylmethanesulfonyl fluoride

(PMSF), 10 mM DTT. It was then ground thoroughly using a pre-chilled mortar and pestle. The solution was transferred to a 1.5 ml Eppendorf tube and spun at 12000g for 2 mins. The supernatant was collected and the protein concentration was determined using the Bradford assay (Bradford, 1976). Samples were then frozen and stored at -80^{o} C.

2.20 Preparing of sample for SDS-PAGE electrophoresis and polyacrylamide gel production.

Before loading the samples onto polyacrylamide gels, the protein samples were heated for 10 mins at 70^{o} C to ensure correct migration on the gel.

Polyacrylamide gels were produced as follows:

Resolving gel: 3.8ml of 1.5M TRIS-HCl (pH 8.8), 6ml of acrylamide (30% w/v acrylamide 0.8% w/v bisacrylamide, 1.5ml of 10% SDS and 3.7ml of H_2O. Polymerization was achieved through the addition of 150 µl 10% ammonium per sulfate (APS) and 6 µl tetramethylethylenediamine (temed).

Stacking gel: 1mL of 1M tris- HCl [pH 6.8], 1.3 mL of acrylamide-bisacrylamide mix, 5.4 mL of H_2O, 800 µL 10% SDS. Polymerisation was achieved through the addition of 150 µl 10% ammonium persulfate (APS) and 6 µl temed.

2.21 SDS-PAGE gels.

The gels were inserted into the Bio-Rad electrode assembly and the tank was filled with running buffer (25 mM tris-HCl 192mM glycine and 0.1% (w/v) sds). Protein samples were loaded into the gels and molecular weight markers were used to estimate the protein molecular weight. The gels were run for 100 mins at 100v.

2.22 Protein transfer.

Once the running of the gels was complete they were removed from their casting plates and placed in transfer buffer (25mM TRIS-HCl, 192 mM glycine and 20% (v/v) methanol. A transfer sandwich composed of sponge, blotting paper, protein gel, PVDF membrane, a further sheet of blotting paper and another sponge was constructed to transfer the protein in the gel to a PVDF membrane. All items were soaked in transfer buffer and placed in the Bio-Rad transfer system ensuring that the PVDF membrane was on the positive (red side) of the tank. The voltage was set to 110v for one hour.

2.23 Immunoblotting.

Once transfer was complete, the PVDF membrane was blocked for 1 hr in 6% skimmed milk (Marvell) in phosphate buffered saline (PBS containing of 137mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2mM KH_2PO_4).

The membranes were washed in a solution of PBS-Tween 20 (0.05% v/v) twice for 30 mins each time on the shaker table and then incubated with the primary antibody for 1 hr and washed in PBS three times for 30 mins. Next, the membrane was incubated in a solution of containing PBS-Tween 20 (0.05% v/v), non-fat dry milk (1.5 % w/v) and the secondary conjugated antibody (1:2000) for 1 hr. Once complete the membrane was washed with PBS three times for 30 mins.

The membranes were transferred to a clean petri dish and soaked with enhanced chemiluminescence (ECL) solution (Amersham) for 2 min. The excess solution was drained and the membranes were placed in a plastic sleeve which was placed in an

autoradiography cassette. The film was then exposed for various times until a suitable result was achieved.

To identify variation in protein loading volumes, PVDF membranes were immersed in a 0.5% Red-Ponceau solution for 2 min and then washed with water until a satisfactory result was obtained.

2.24 Detection of starch.

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

2.25 Transketolase activity assay.

In plants the protocol typically used to measure TK activity is where the substrates Xu5P and E4P are utilized to trigger the following reaction (Gibon et al., 2004):

Xu5P+E4P >**TK**> F6P+G3P

This reaction results in the oxidation of NADH to NAD+ causing a decrease in absorption at 340nm.

Due to the current unavailability of xylulose-5-phosphate an alternative method was sought to measure TK activity. One method identified was that used in erythrocytes where TK activity is measured through the reaction: Xu5P+R5P >TK> S7P+G3P (Figure 2.1).

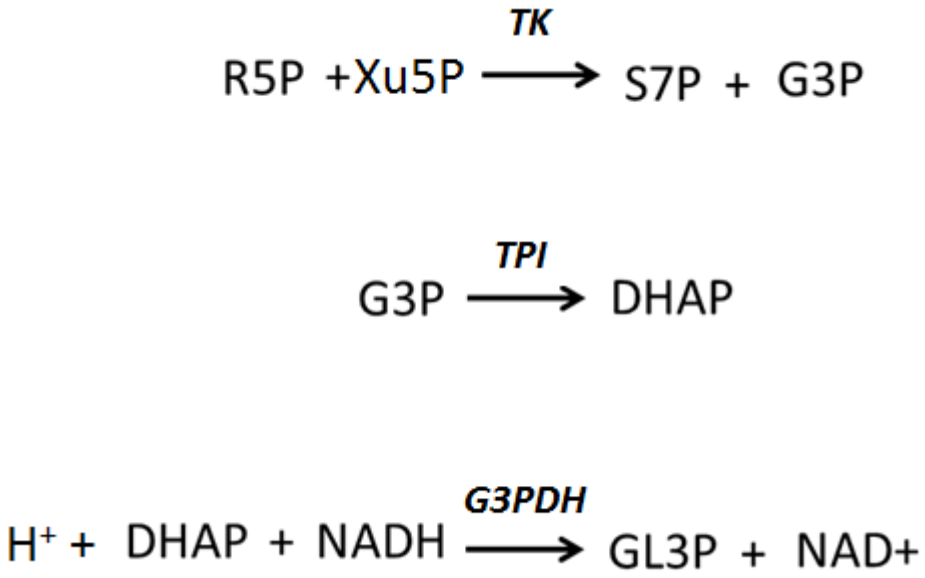

Figure 2.1- TK activity reaction resulting in the oxidation of NADH: A two carbon fragment is removed from xylulose-5-phoshate (Xu5P) by transketolase (TK) and added to ribose-5-Phosphate (R5P) creating seduheptulose-7-phosphate (S7P) and glyceraldehyde-3-phosphate (G3P). G3P is then converted to dihydroxyacetone-phosphate (DHAP) by the enzyme triose phosphate isomerase (TPI). In the final step DHAP is converted to glycerol 3-phosphate (GL3P) by glycerol-3-phosphate dehydrogenase (G3PDH) which oxidises NADH to NAD+ causing a decrease in absorbance at 340 nm.

This assay depends on the conversion of a portion of the R5P substrate to Ru5p and then to Xu5P by the endogenous ribose phosphate isomerase and ribulose phosphate epimerase, respectively, which are present in the crude leaf extracts. This process triggers a reaction cascade which results in the oxidation of NADH to NAD+ causing a decrease in absorption at 340nm (Zhao.Y 2014). These absorbance values are measured at 5 minute intervals for one hour. Using the change in absorbance values over a time period where the oxidation of NADH is linear, this allows the calculation of total protein activity per minute.

Because Xu5P is not used in the reaction mix it is therefore derived from the enzymes, ribose-5-phosphate isomerase (Rpi), which forms RUBP from R5P, and ribulose 5-phosphate epimerase (R5Pe), which forms the Xu5P from R5P. Though the levels of these enzymes in plants is unknown it has been shown that they are non-limiting for this reaction in erythrocytes (Bruns et al., 1958). In addition, when using this technique in plants the possibility exists that intermediates from the Calvin cycle may be fed into the reaction, leading to an incorrect result.

Using solely R5P as a substrate it was found that this protocol gave comparable results on a proportional basis compared to those shown previously (Khozaei, 2010) in a TK activity assay that utilizes Xu5P and E4P as substrates (Figure.2.2). The results were comparable with the level of total TK protein found in protein quantification analysis (Figure.2.3).

Whilst the rate of reaction was found to be directly proportional to the concentration of the total protein extract (Figure.2.4) added to the reaction and only a slight variation (within 6%) was seen in technical replicates. Negative controls identified that the addition of R5P to the reaction, which is both a product and an intermediate of the

Calvin cycle and the pentose phosphate pathway is key to the reaction taking place (Figure.2.5)

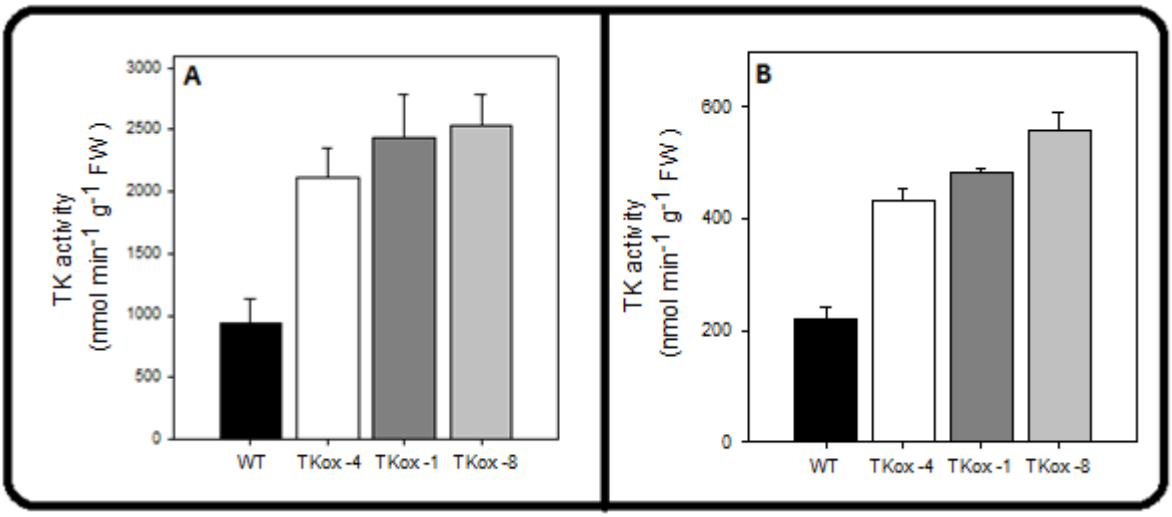

Figure 2.2- The comparison of TK activity using different methods on comparable plant material. Due to the unavailability of xylulose-5-phopshate an alternative transketolase activity method was required. (A) TK the activity level using the XU5P and E4P method (Gibon et al., 2004) by (Khozaei, 2010). (B) TK activity using only R5P as a substrate (Zhao.Y 2014). B is replicated here from chapter.3 (Figure 3.8) as a comparison of results using a different enzyme activity assay to that used previous.

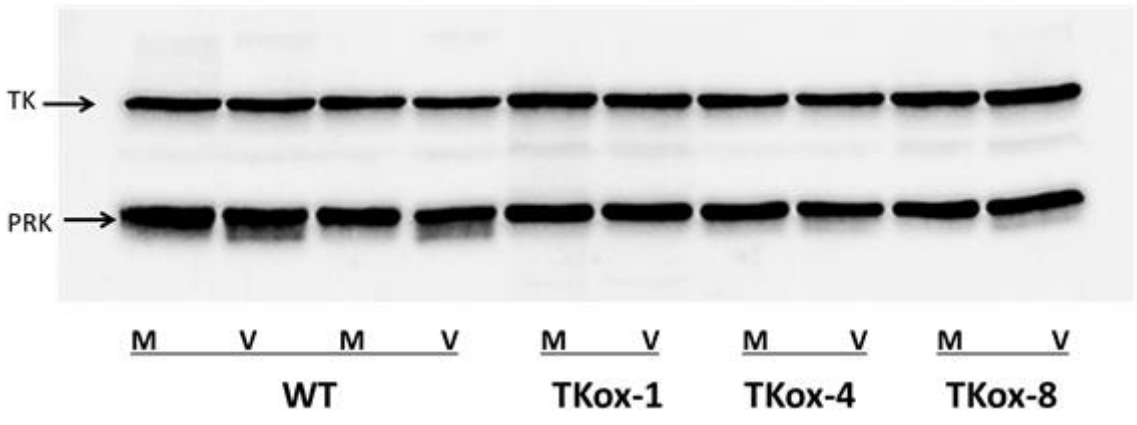

Figure 2.3- The levels of transketolase (TK) and PRK in WT and TKox lines (-1, -4 and -8). Protein was extracted from the vein and mesophyll tissue newest fully expanded leaf of WT and TKox lines and the resulting samples were separated on a 12% acrylamide gel and analysed by immunoblotting using polyclonal antibodies raised against native TK and PRK. Figure partially replicated from chapter.3 (Figure 3.5) to show comparative protein levels to TK activity)

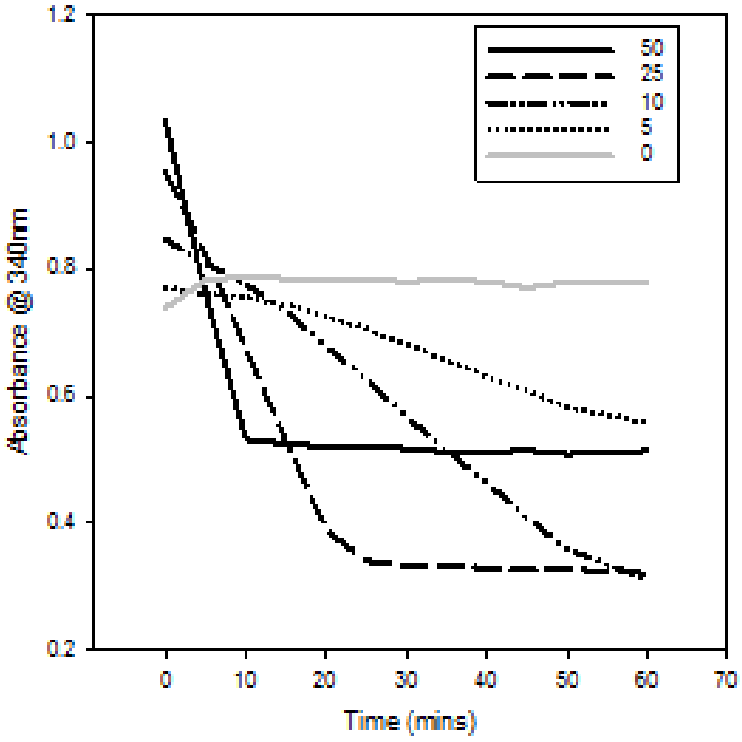

Figure 2.4 -Oxidation of NADH falls as total protein levels are decreased. The fall in absorbance in relation to the amount of total protein extract added to the TK activity reaction. Volumes of 50, 25, 10, 5 and 0 µl of 600 ng/µl^{-1} total protein solution were added to the TK reaction mix. Where volumes were lower, extra buffer was added to ensure all reactions contained the same total volume.

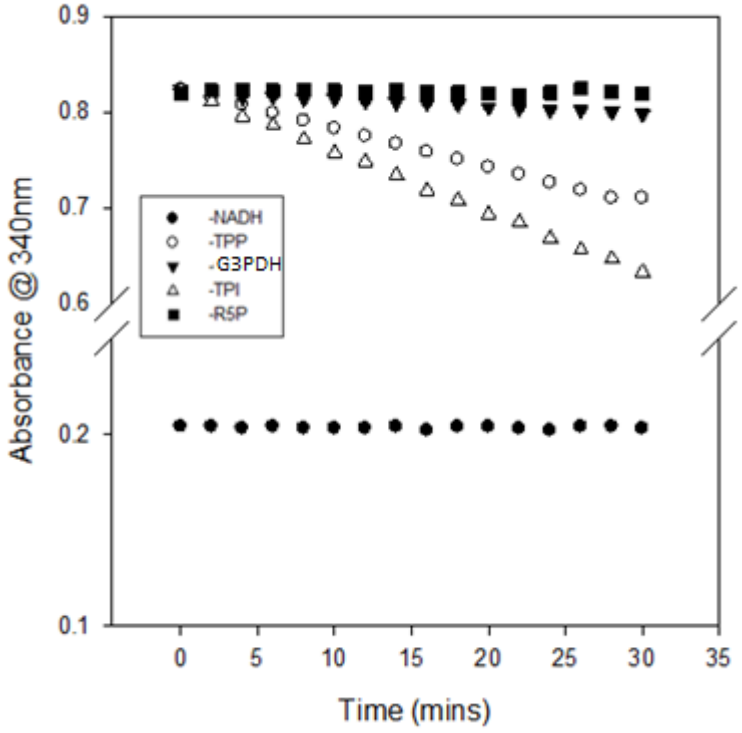

Figure 2.5- Negative controls for the TK activity reaction. Reaction mixes were produced that were absent of the following enzymes/ substrates: NADH (nicotinamide adenine dinucleotide), TPP (thiamine pyrophosphate), G3PDH (glycerol-3-phosphate dehydrogenase), TPI (triose phosphate isomerase), R5P (ribose 5 phosphate). Changes in absorbance at 340nm which decreases when it is oxidised to NAD+ were monitored for 30 minutes.

2.26 TK activity protocol used in this study

Plant tissue was snap frozen in liquid nitrogen and then ground to a fine powder. Approximately 50 Mg of this powder was added to 300 µl of ice-cold 0.1 M TRIS-HC1 buffer (pH 7.6) containing 2 mM benzamidine, 2 mM aminocaproic acid, 0.5 mM PMSF and ground using a chilled mortar and pestle and then spun at 10000g for 3 mins. 165 µl of the supernatant was removed and stored in a new Eppendorf tube and kept on ice. 15ul of this was used to quantify protein levels using the Bradford method (Bradford, 1976) and diluted with ice-cold 0.1 M TRIS-HCl buffer (pH 7.6) to give a total protein concentration of 600 ng/µl^{-1}. 230 µl of pre-prepared assay mix comprising of: 14.4 Mm ribose-5-phosphate, 190 µM NADH, 380 µM TPP, 250 U/L^{-1} glycerol-3-phosphate dehydrogenase (G3PDH) and 6500 U/L^{-1} triose phosphate isomerase was transferred to a 96 well plate (Greiner Bio-One) and placed in a plate reader which was set at 23^{o} C for 5 minutes. The plate was then ejected and protein samples of 20 µl each were injected into the wells containing the assay mix. The plate was inserted back into the plate reader was then read for absorbance at 340 nm every 5 mins for 1 hr. Activity levels were estimated by subtracting the absorbance value when the reaction becomes linear from the absorbance value 20 to 30 minutes after the first absorbance reading depending on the rate of the reaction.

This value was plugged into the equation produced from an NAD+ standard curve. The number produced is then divided by the amount of minutes passed between the

first and second absorbance readings which gives the amount of NADH oxidized per minute.

2.27 HPLC measurements

2.28 Thiamine extraction technique

From seed:

100 Mg of seed were analysed for their thiamine/TPP content in the following conditions: dry, imbibed in water, thiamine or TPP. All seeds were imbibed for 24 hours at 4^{o}C then washed in purite water (SUEZ environment) five times, dried, and then stored at -20^{o} C until extraction. Dry seeds were also stored at – 20^{o} C.

From leaf material:

Thiamine and TPP were extracted as shown in Kozik,1995 with some modifications. 300 Mg of leaf material was snap frozen in liquid nitrogen, crushed and then homogenised in 1 ml of extraction buffer (50 mM potassium phosphate buffer (pH 7) containing 0.15 M NaCl, 1 mM phenylmethylsulphonyl fluoride (dissolved in DMSO), 1 mM EDTA, 1 mM dithiothreitol, 1 % polyvinylpyrrolidone and then transferred to a 1.5 ml Eppendorf and centrifuged at 4^{o} C for 30 mins at 14000 G. 500 µl of the supernatant was carefully removed and added to 250 µl of 10 % acetic acid. This was mixed and put on ice. It was left for 10 mins before being centrifuged at 4^{o} C for 10 mins at 6000 G. 500 µl of the supernatant was transferred into a new 1 ml Eppendorf. The pH of the solution was adjusted to ~ pH 5 through the addition of ~ 30 µl of 3M sodium acetate.

2.29 Pre-column derivitisation of Thiamine.

Measurement of thiamine and its phosphate esters using HPLC requires the derivitisation of thiamine to thiochrome (Lu and Frank, 2008). Thiochrome is formed by oxidation of thiamine which causes the product to emit blue fluorescence under ultraviolet light that serves as the basis for determining individual thiamine and TPP concentrations. It uses hexacyanoferrate III as a derivatising agent. This was prepared by mixing 15 % NaOH (W/V) and 1 % potassium ferricyanide (W/V) in a 9:1 ratio. These separate solutions were freshly prepared before each sample run and stored at 4^{o} C. For each run of thiamine measurements the two chemical solutions were mixed and used immediately.

The derivatising agent and thiamine plant extracts were prepared in a 96 well plate in the dark. 25 µl of derivatising agent was added to the required wells and then 40 µl of the thiamine extract was pipetted into each well and mixed. The plate was sealed to prevent evaporation of sample causing an increase in concentration and then placed in the HPLC machine injector/sampling unit kept at a temperature of 10^{o} C. In testing, this technique provided derivatised samples are stable for up to 24 hours with a variation of no more than 5% in fluorescence values.

2.30 HPLC analysis of oxidized thiamine products.

Thiochrome products were analysed using an Agilent 1100 series HPLC system using reverse phase chromatography. Separation was achieved using a Phenomenex Luna C18 analytical column (150 x 4.6mm, 5µm) protected with a 4.6 mm guard cartridge. The Injection volume was 20 µl and the column temperature was set at 25^{o} C whilst the auto sampler temperature was set at 10^{o} C. Fluorescence excitation of the injected solution was set at 375 nm whilst emission was 435 nm. Between each injection the needle was washed in water and the column was allowed to equilibrate. 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 0-2 mins of 100 % phase (a) then from 2-12 mins mobile phase (B) increased to 100 % which was held for a further 8 mins. From 20-24 mins 100 % of mobile phase (A) was used to prepare the column for the next sample. Peak analysis was conducted using the Agilent Chemstation software. Standard curves of both thiamine and TPP were produced (Figure.2.6)

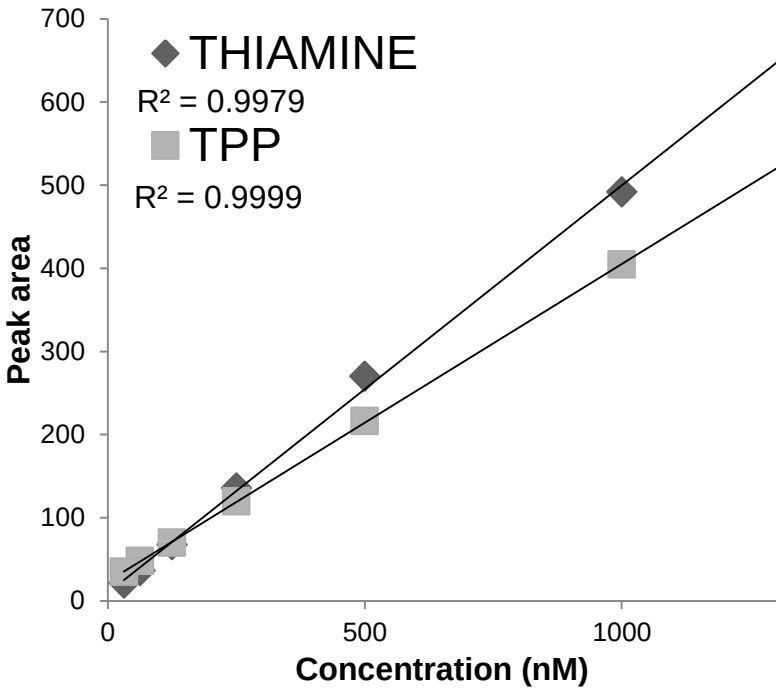

Figure 2.6-Standard curves of Thiamine and TPP using reverse phase Chromatography: Known quantities of thiamine and TPP were prepared in a 1% TCAA solution to the expected concentrations to produce a standard curve.

2.31 Statistical analysis.

All statistical analyses were performed using one-way analysis of variance (ANOVA), Tukey's post hoc test in R v.2.6.2 (http://www.r-project.org). Significant differences between transgenic lines and wild-type for the different parameters evaluated are reported with asterisks (* = $P<0.05$) on figures.

CHAPTER 3

Molecular and physiological characteristics of transgenic tobacco with increased transketolase activity

Introduction

The Calvin Benson (C_3) cycle is the primary pathway of atmospheric CO_2 uptake and fixation into organic molecules. Studies of the C_3 enzyme sedoheptulose-bisphosphatase (SBPase) (Harrison et al., 2001) (Lawson et al., 2006) (Lawson et al., 2008) showed that changes in the activity level of a solitary C_3 enzyme can increase growth rate and yields. Metabolic control analysis identified that TK transketolase (TK) has significant control over the C_3 cycle and changes to its activity level may also improve growth.

To confirm this, antisense TK tobacco plants were produced. It was discovered that a decrease in TK activity of between 20-40 % was found to limit photosynthesis and decrease the levels of aromatic amino acids and phenylpropanoids. This finding was attributed to reduced availability of erythrose 4 phosphate (Henkes et al., 2001). Comparison of these results with anti-sense studies of the C_3 enzymes SBPase and FBP aldolase plants showed that the changes seen were unique to TK and are not due to a reduction in carbon availability from the C_3 cycle (Haake et al., 1998; Harrison et al., 1998; Haake et al., 1999). It was thought that an increase in TK activity may produce a beneficial effect as previous studies had shown the increase of a solitary C_3 enzyme in SBPase was able to increase biomass (Lefebvre et al., 2005).

Therefore a TK sense mutant was produced which comprised of a full-length Arabidopsis TK3 (At3g60750) cDNA that was used to prepare a sense gene construct

in the binary vector pRoK2 containing the cauliflower mosaic virus (CaMV) 35S promoter and NOS terminator.

In the initial analysis of these plants it was identified that an increase in TK activity levels of ~2 times that of WT caused a chlorotic and growth phenotype (Lefebvre, 2005). Further research showed that the phenotype was due to an unidentified mechanism involving changes to thiamine metabolism (Khozaei, 2010). If TK sense plants were germinated in a thiamine enriched media this was sufficient to rescue both the growth and chlorotic phenotypes.

The aim of the work in this chapter is to explore the impact of thiamine availability from the seedling stage through to mature plants by investigating its effect on growth and development as well as the molecular and physiological characteristics of Tkox lines.

Results

TKox lines had previously been identified as having a growth and chlorotic phenotype that was able to be rescued via thiamine or TPP supplementation at the point of germination (Khozaei, 2010). However, the reasons as to why an increase in TK protein levels would alter thiamine metabolism were not identified. This research aims to identify the reasons for the changes to thiamine metabolism.

However, prior to exploring the reasons for the phenotype, a thorough characterization of the TK overexpressing lines was conducted to identify variations in the levels of TK activity and the effect this had on growth and development, protein levels of other Calvin cycle enzymes levels and carbon allocation.

3.1 TKox lines exhibit a growth and chlorotic phenotype that correlates with the increase in TK protein levels.

WT plants and three TKox lines were germinated in Murashige and Skoog medium (MS) and grown in long day conditions in a temperature controlled environment with light levels of 200-250 mmol m^{-2} s^{-1} PPFD and a temperature of 25º C. At 12 days post germination the plants were transferred to soil and at ~25 days they were moved to greenhouse conditions for the remainder of their lifecycle.

The TKox lines (-1, -4 and -8) all exhibited a slow growth and chlorotic phenotype (Figure.3.1).The pattern of chlorosis showed two main variations (Figure.3.2). One was found around the perimeter of the leaf whilst the other extended throughout the mesophyll regions of expanding true leaves. In seedlings, the chlorotic phenotype was only found on the cotyledons whilst growth rates in TKox lines showed significant decreases compared to WT plants. TKox lines -1,-8 were shown to be the most severely affected (Figure.3.3)

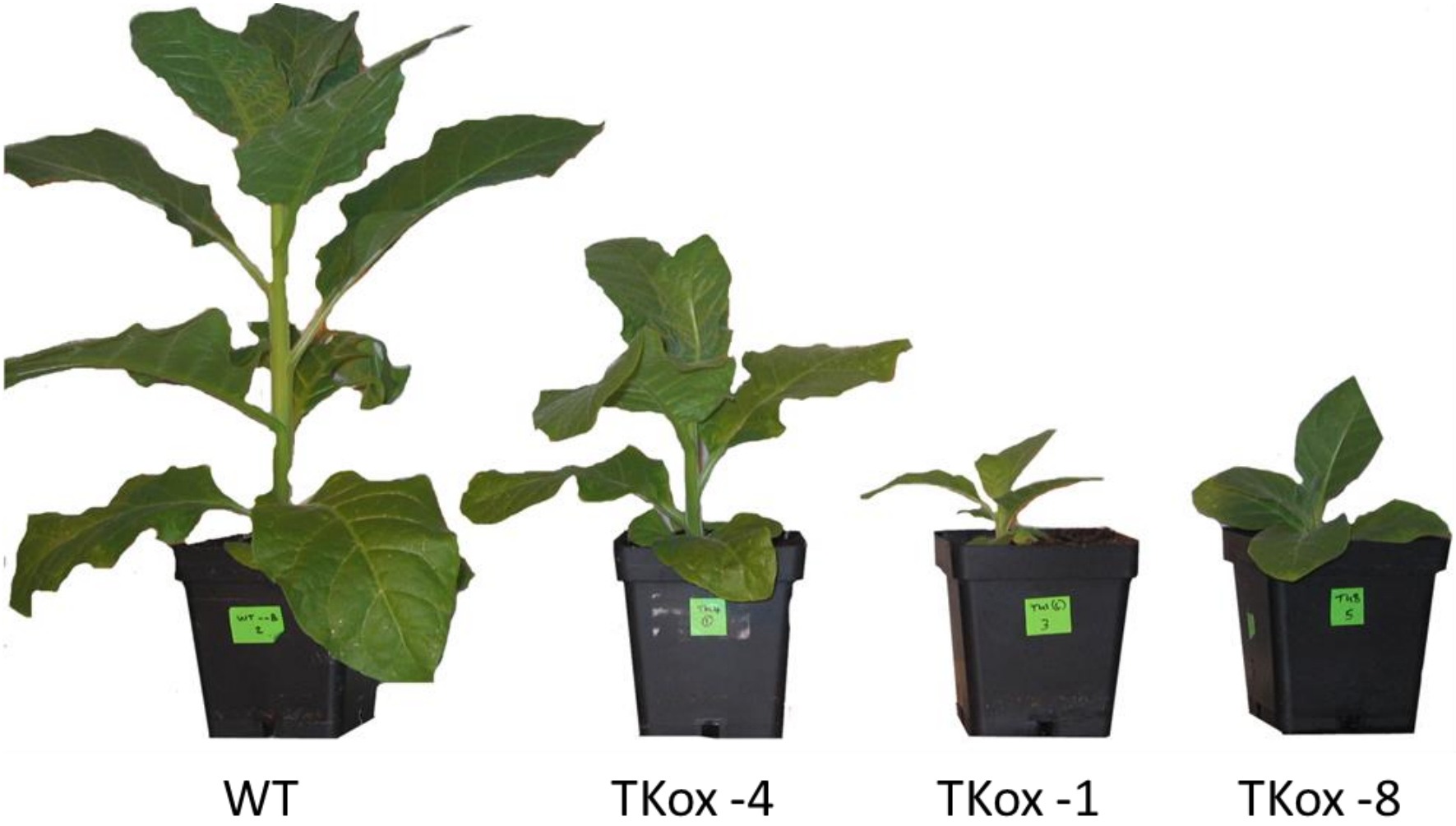

Figure 3.1- Growth Phenotype of Tkox lines. Transgenic TKox and wild-type plants were grown in green house conditions under light levels of between 600 and 1500 µmol m^{-2} s^{-1}. Photograph was taken at ~5 weeks post germination.

Figure 3.2- The chlorotic phenotype of Tkox lines. Tkox Plants show chlorosis of both the leaf perimeter and mesophyll regions.

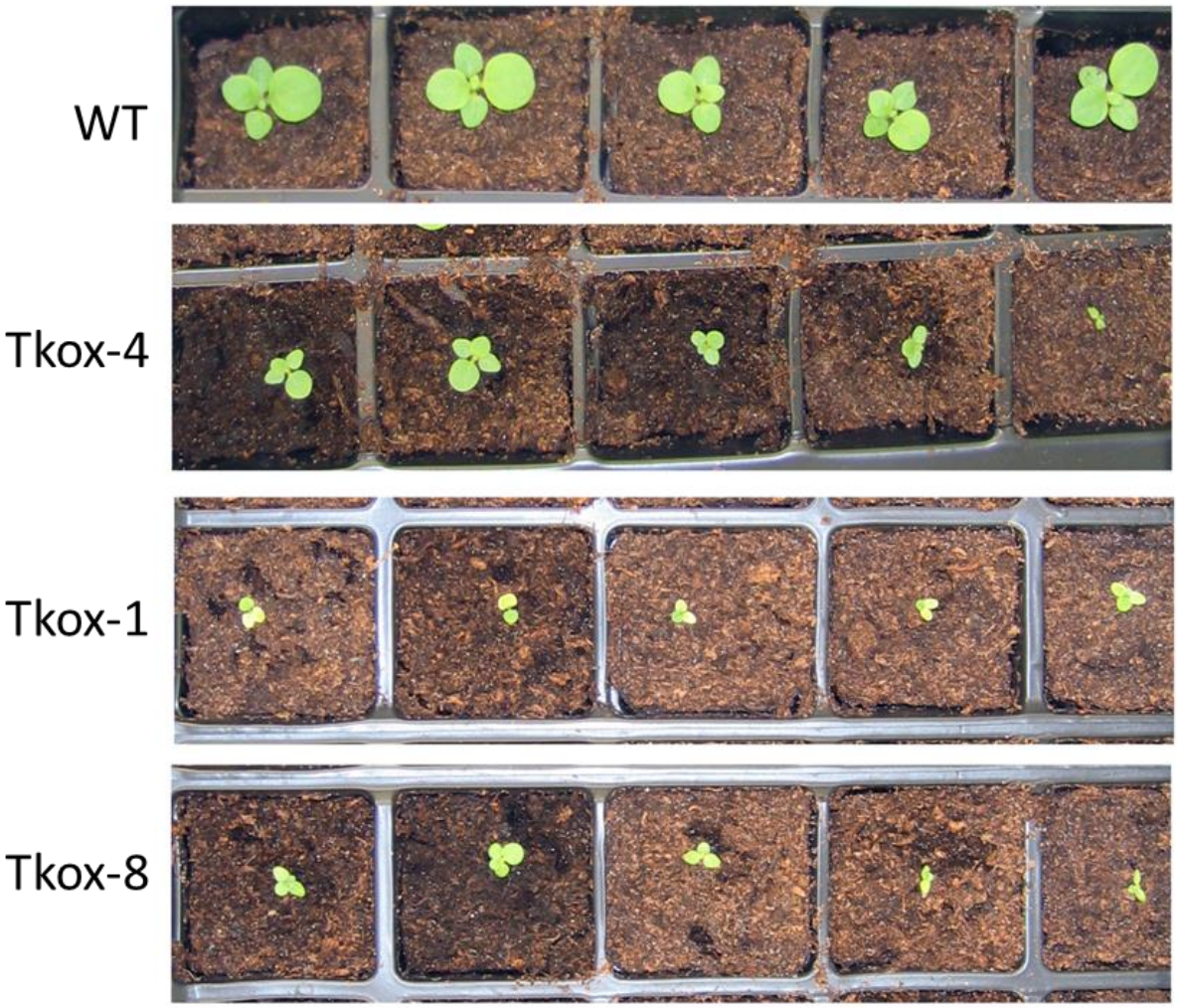

Figure 3.3- Phenotype of Tkox seedlings. Transgenic Tkox line -4,-1, -8 and wild-type plants were grown long day conditions under light levels of 150 µmol m^{-2} s^{-1}, 25°C. Photograph was taken at 16 days post germination.

3.2 Tkox exhibit a growth and chlorotic phenotype that correlates with the increase in TK protein levels.

To identify whether the severity of the phenotype showed a relationship to the level of TK protein seen in the TKox lines an immunoblot analysis was conducted (Figure.3.4). It was found that TK protein levels were increased between 2 to 2.5-fold that seen in WT plants and the highest levels of TK protein were shown to be in the Tkox -1 and -8 lines. These lines were shown to have the most severe phenotypes. Immunoblotting also showed that the TKox lines had increased levels of the deoxy-xylulose synthase (DXS) protein but did not show any significant change in protein levels for the C_3 enzyme phosphoribulokinase (PRK). A qPCR analysis of the same material confirmed high levels of expression of the introduced transgene in the lines Tkox -1, -4 and -8, with 10-28 fold more transcripts for the *Arabidopsis TK* (At-TKL) than the endogenous tobacco *TK* (Nt-TK) (Figure.3.5).

To identify whether there was co-suppression of the native *TK* gene a qPCR analysis was conducted using primers that were specific for the plastid *TK* (ACF60500.1) gene transcripts (Figure.3.6). Transcript levels were found not to significantly differ from WT showing that there was no evidence of co-suppression of the native *TK* genes by the high level of the introduced At-TKL transcripts.

To determine the in-vitro level of TK activity total protein samples were assayed in optimal conditions. It has been shown that such measurements provide a proxy for protein abundance (Piques et al., 2009). The TK activity data confirmed the increases in TKox lines observed using immunoblot analysis (Figure.3.7).

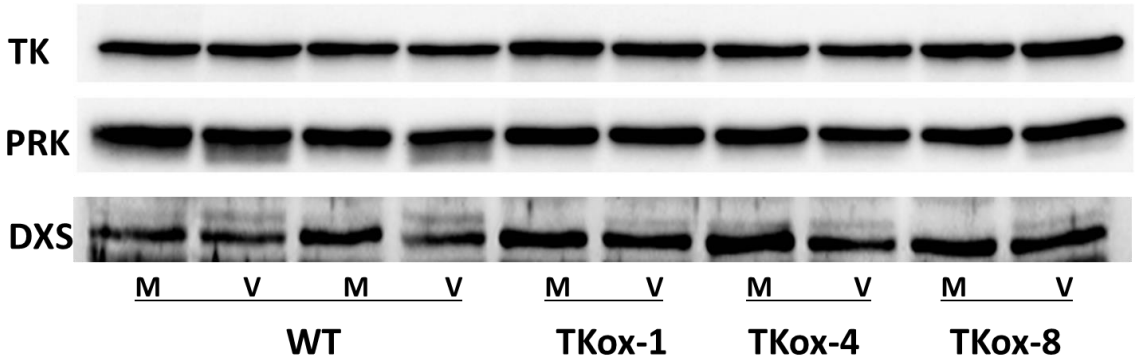

Figure 3.4- Transketolase (TK) 1-deoxy-D-xylulose-5-phosphate synthase (DXS) and phosphoribulokinase (PRK) protein levels in TKox and WT plants. Immunoblot analysis of protein extracts from the newest fully expanded leaf of TKox and WT plants from (V) vein and (M) mesophyll material. Proteins (5 µg) were separated on a 12% polyacrylamide gel and blotted onto PVDF membrane and probed with polyclonal antibodies against TK, DXS and PRK proteins.

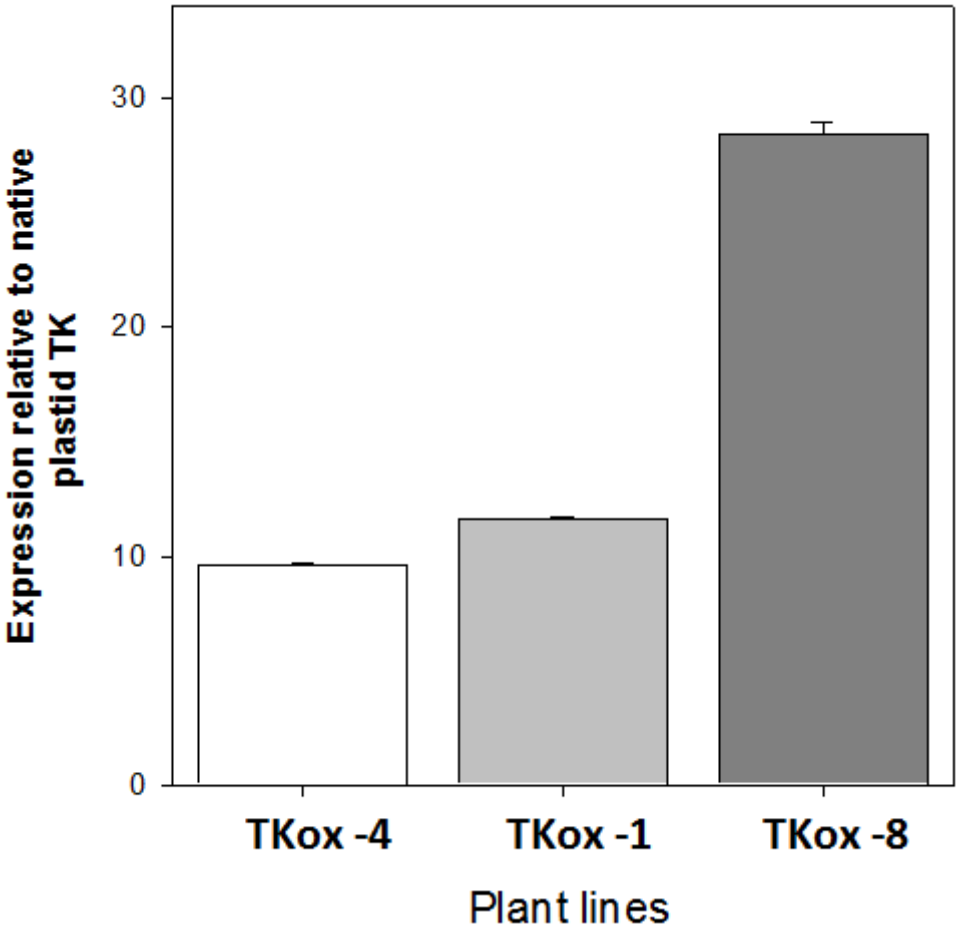

Figure 3.5- The level of introduced plastid TK transgene transcripts compared to native TK transcripts. qPCR analysis of the introduced plastid TK transgene compared to the native tobacco plastid transketolase gene (ACF60500.1). The values represent a pooled sample of RNA from 4 independent plants from each group. The results are the mean from 3 technical replicates and the error bars indicate the S.E.

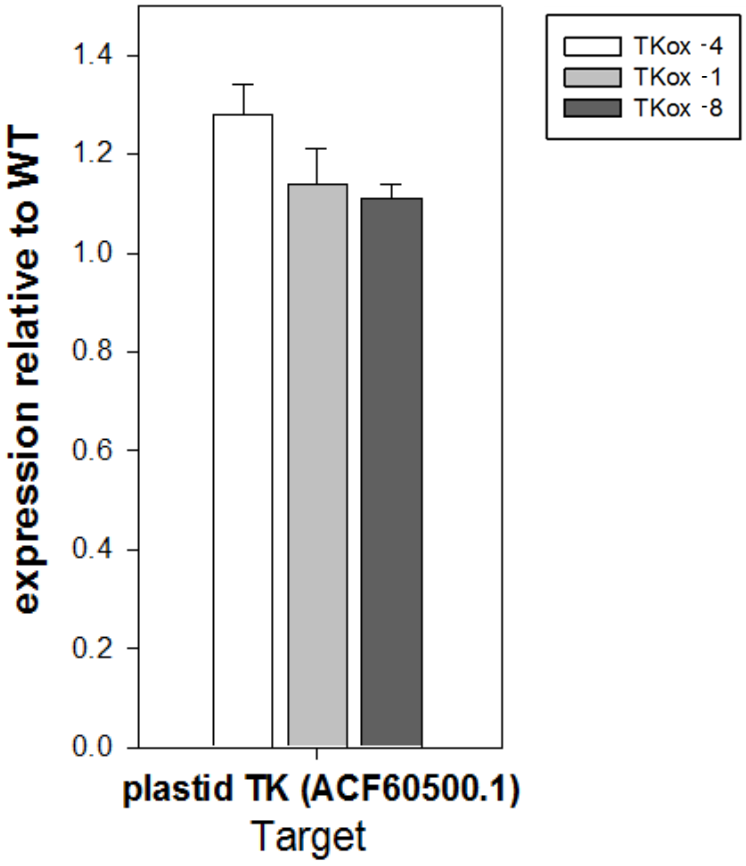

Figure 3.6- Relative transcript level of native plastid TK in Tkox lines compared to WT. The values represent a pooled sample of RNA from 4 independent plants from WT and TKox lines -1 -4 -8 that was used to produce cDNA to be used in qPCR reaction. The error bars indicate the S.E of 3 technical reps.

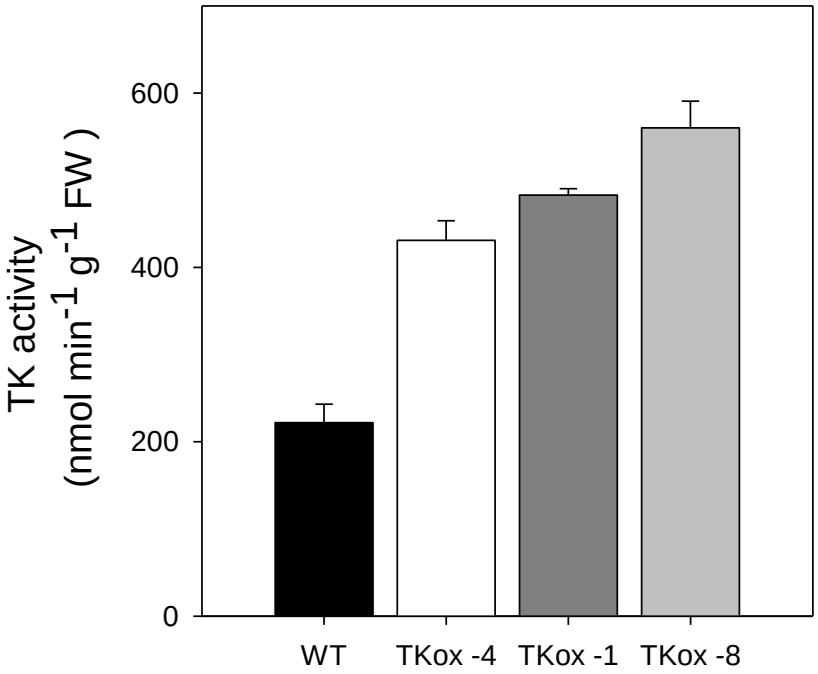

Figure 3.7- The level of TK activity in WT and Tkox lines. Total protein extracts from the newest fully expanded leaf of TKox and WT plants were diluted to 600 ng μl^{-1}. 20 µl of this solution was added to the assay buffer which was monitored for changes to its absorbance at 340nm to determine TK activity levels. The results are the mean from 4 biological replicates and the error bars indicate the S.E. This figure is repeated in chapter as an example of the results using this method compared to those used by (Khozaei, 2010)

As the 35S CaMV promoter is known to provide constitutive expression of the gene under its control it was decided to investigate whether total TK protein levels were also increased in the root system. An immunoblot analysis was conducted on 12 day old seedlings that had been separated into their root and shoot systems (Figure.3.8). The results showed that TK protein levels in WT plants was negligible whilst the TKox lines tested showed substantial levels of total TK protein

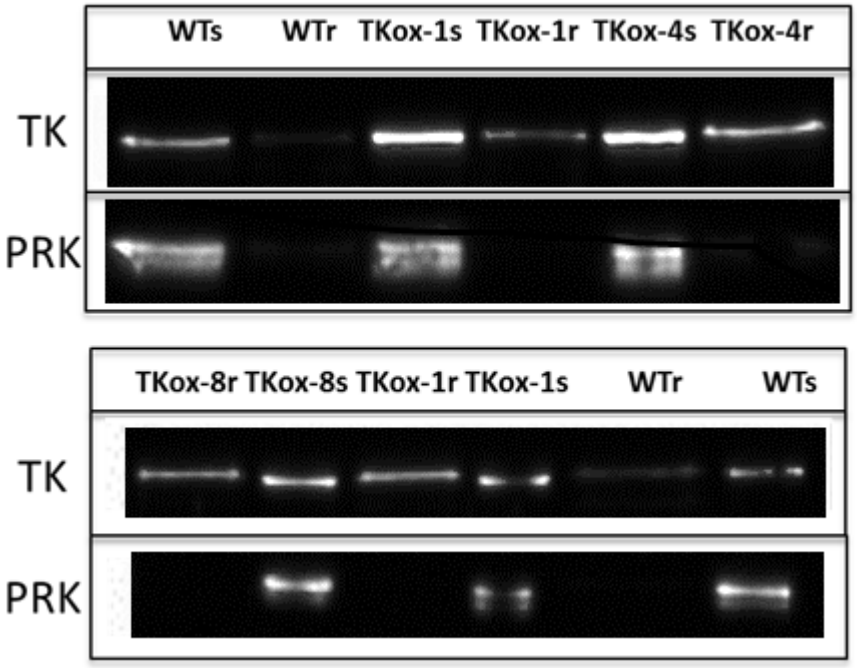

Figure 3.8- The levels of transketolase (TK) and phosphoribulokinase (PRK) in the shoot and root system of 12 day old WT and Tkox lines. Protein was extracted from the root and shoot systems of 12 day old seedlings leaf of WT and TKox lines -1 -4. The resulting samples were separated on a 12% acrylamide gel and analysed by an immunoblot using polyclonal antibodies raised against native TK and PRK. Postfix symbols after line indicate: r(root tissue) and s(shoot tissue). The results shown are for two pooled biological replicates for each of the TKox lines and WT plants.

3.3 Imbibition of seeds in thiamine is sufficient to rescue both the growth and chlorotic phenotypes.

TKox lines have previously been found to exhibit a growth phenotype that was rescued if seeds were germinated in a thiamine or TPP enriched media. However, because thiamine is stored in the seed and subsequently used in the establishment of newly germinated seedlings, this raised the question as to whether it was a shortage of thiamine stored in the seed that was causing the phenotype. To test this, Tkox seeds were imbibed in a thiamine solution, washed in water and then germinated on thiamine deficient media (Figure.3.9). The results showed that the imbibition of seeds in thiamine was sufficient to allow TKox lines to produce root and shoot systems that were comparable to WT plants.

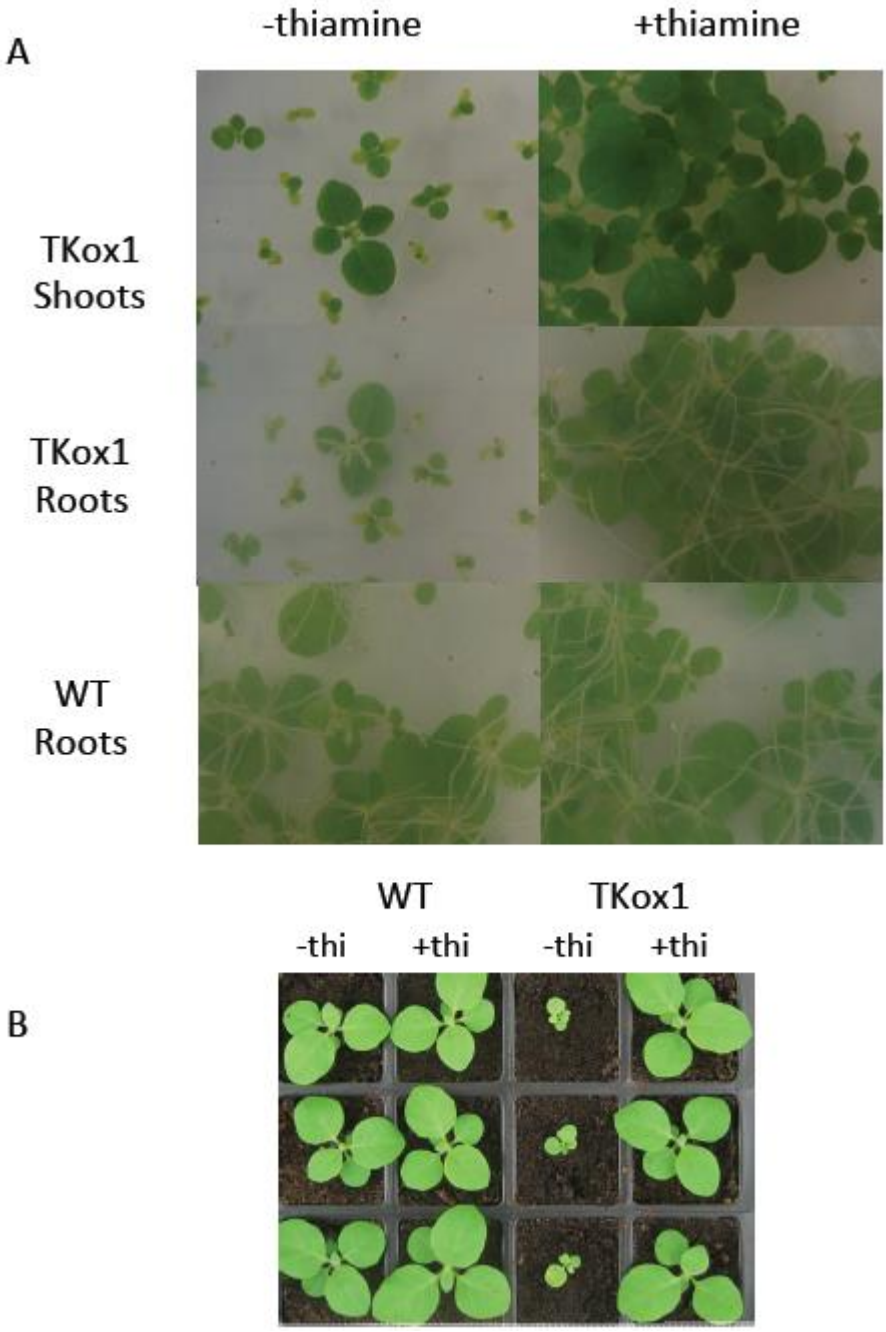

Figure 3.9- Imbibition of Seeds of TKox in water supplemented with thiamine is sufficient to complement the growth phenotype. Seeds of WT and Tkox -1, -4 plants were imbibed in water or water plus thiamine (50mg L^{-1}). Seeds were then rinsed in water x3, dried and then germinated on MS media. At 12 days post germination, seedlings were transferred to soil. A. Shoot system of WT and Tkox lines at 20 days post germination. B. Shoot system of 12 day Tkox-1 seedlings.

3.4 The effect of thiamine supplementation on growth and development is dependent on the timing of supplementation.

To determine how thiamine availability would affect the growth of both the root and shoot systems during germination and early plant establishment. WT plants plus TKox lines -1 and-8 were germinated in MS medium with (+) or without (-) thiamine supplementation for a period of 12 days (Figure.3.10). At 12 days post germination the seedlings were transferred to soil and irrigated with water alone or water plus thiamine. It was clear that thiamine availability at the point of germination is essential to recover the growth phenotype as only (++) and (+-) plants had rosette growth was similar to that of WT plants. The importance of thiamine availability at the point of germination in TKox lines was confirmed in a destructive harvest that showed dry weights and leaf areas in TKox lines were as low as 10% of those Tkox plants that received thiamine at the point of germination (Figure.3.11).

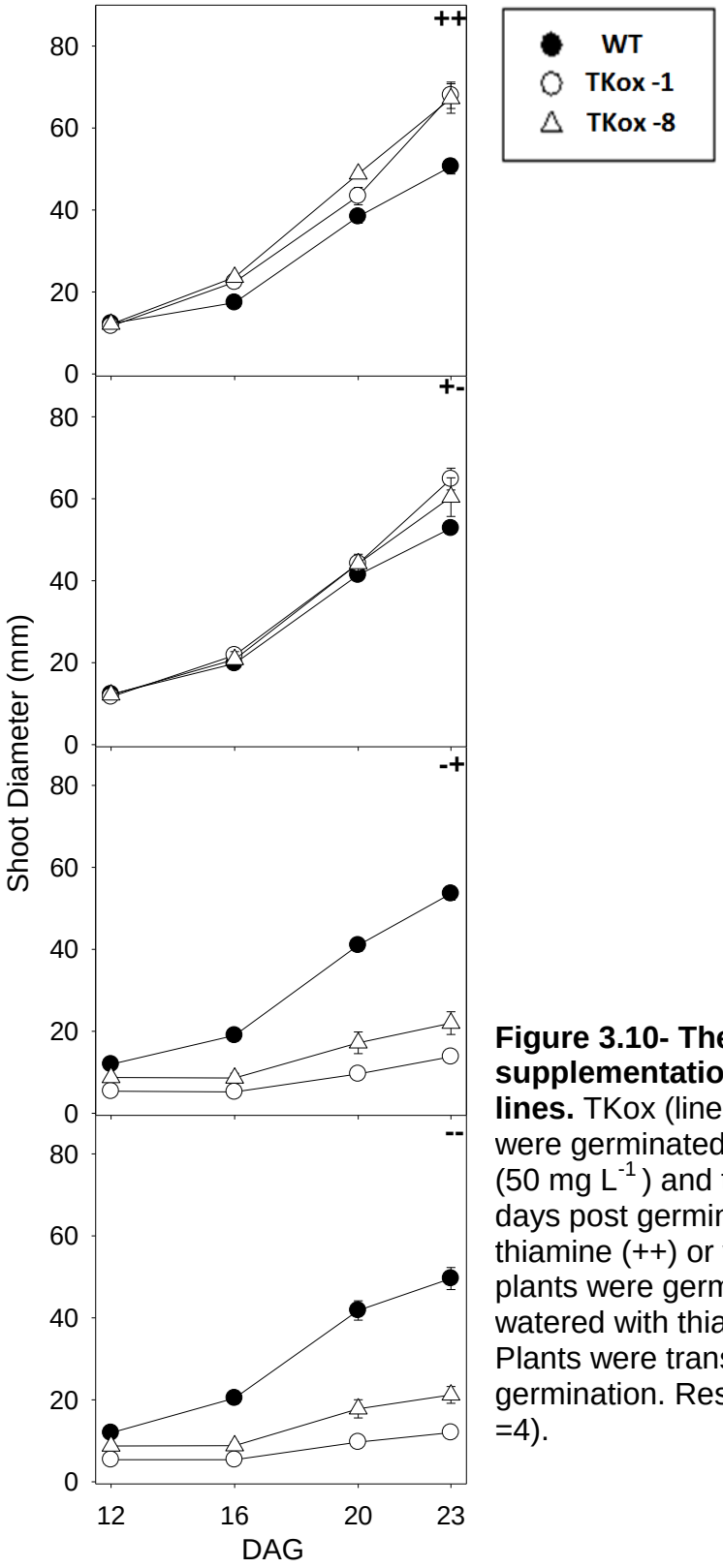

Figure 3.10- The timing of TPP supplementation is critical to rescue TKox lines. TKox (lines -1 and -8) and WT plants were germinated in media containing thiamine (50 mg L^{-1}) and then transferred to soil 12 days post germination and watered with thiamine (++) or without (+-). A second set of plants were germinated without thiamine and watered with thiamine (-+) or without (--). Plants were transferred to soil 12 days after germination. Results are the mean ± SE (n =4).

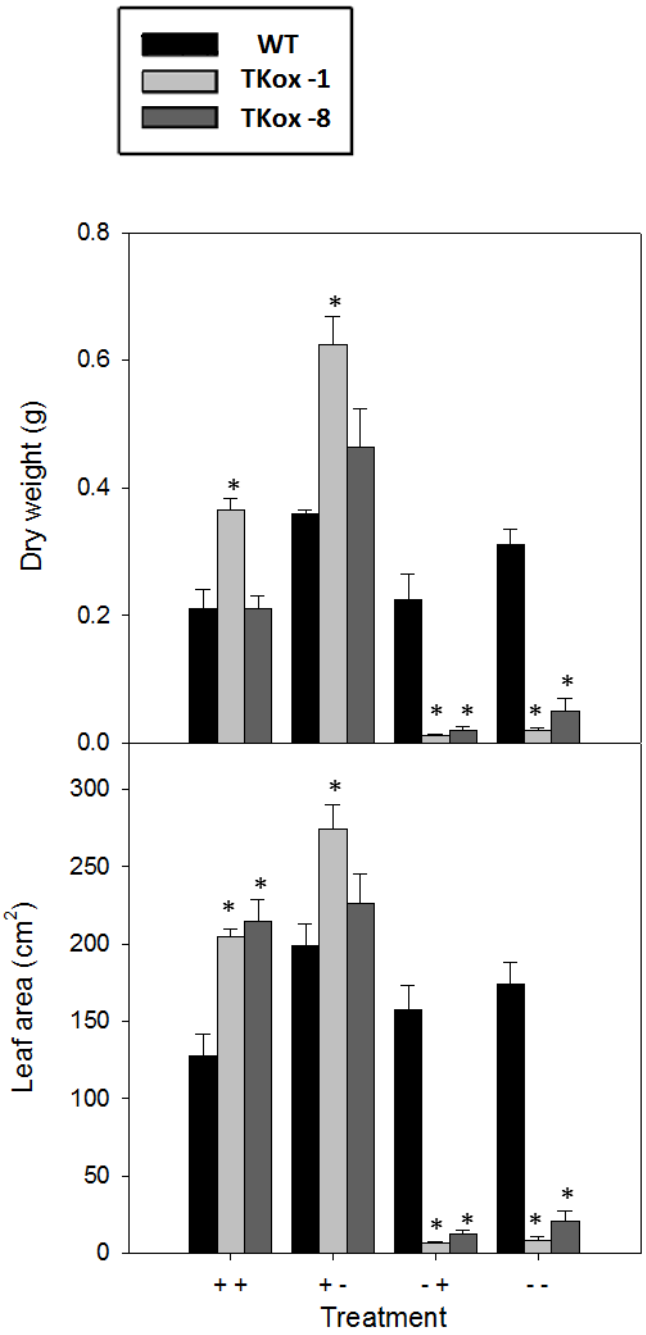

Figure 3.11- Dry weight and total leaf area of TKox and WT plants. Destructive harvest was conducted at 34 days post germination and leaf area and shoot dry weight determined. TKox (-1 and -8) and WT plants germinated in media containing thiamine (50 mg L-1) and then transferred to soil 12 days post germination and watered with thiamine (+/+) or without (+/-). Plants germinated without thiamine and watered with thiamine (-/+) or without (-/-).Results are the mean ± SE (n =4).Statistical comparison was made using a one way Anova (95 % sig) and Tukey post hoc test. * indicates significance at P< 0.05 when compared to WT plants under the same thiamine supplemental regime.

These findings suggested that a more in-depth experiment involving thiamine supplementation and withdrawal would yield a further insight into the thiamine requirements of TKox lines and its effect on their growth and development (Figure.3.12). Seeds from WT and all three TKox lines were germinated and grown as per the previous experiment. Those TKox lines grown in the absence of thiamine (--) displayed a chlorotic phenotype and were smaller than wild type plants. Plants that were germinated without thiamine but were later supplemented with thiamine following their transfer to soil (-+) produced small plants similar to the size of the (--) but the chlorotic leaf phenotype was complemented. Tkox plants germinated in the presence of thiamine which were then transferred to soil and given only water (+-) grew to a size similar to that of the WT plants but exhibited a chlorotic phenotype. This experiment provided evidence that TKox lines require a supplemental source of thiamine at the point of germination whilst the chlorotic phenotype can be rescued at any time if an exogenous thiamine source is available.

3.5 Photosynthetic carbon assimilation is reduced in plants that exhibit the chlorotic phenotype.

To determine whether thiamine supplementation can affect the levels of carbon assimilation in Tkox lines compared to WT the 6th fully expanded leaf of plants grown under the thiamine supplementation regime as discussed previous was used to identify the carbon assimilation rate under varying CO_2 conditions (Figure.3.13). As expected, those plants which exhibited the chlorotic phenotype (-- and +-) showed a significant fall in assimilation rate whilst the (-+) and (--) TKox lines exhibited assimilation rates not dissimilar to WT plants.

Figure 3.12- Timing of thiamine application determines the presence of the growth and chlorotic phenotypes. TKox and WT plants were germinated in media containing thiamine (50 mg L^{-1}) and then transferred to soil 12 days post germination and watered with thiamine (++) or without (+-). A second set of plants were germinated without thiamine and watered with thiamine (-+) or without (--). Photograph was taken after 34 days in soil.

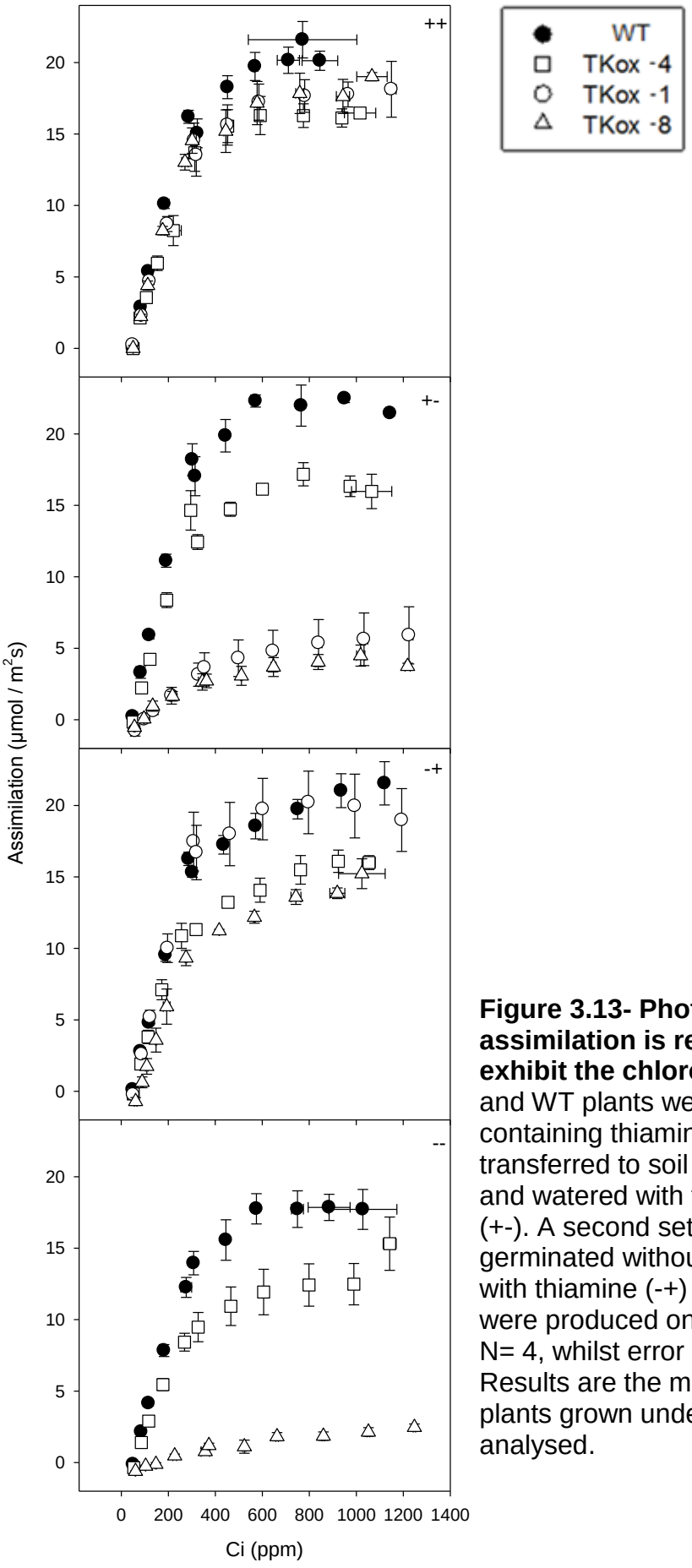

Figure 3.13- Photosynthetic carbon assimilation is reduced in plants that exhibit the chlorotic phenotype. TKox and WT plants were germinated in media containing thiamine (50 mg L^{-1}) and then transferred to soil 12 days post germination and watered with thiamine (++) or without (+-). A second set of plants were germinated without thiamine and watered with thiamine (-+) or without (--). Aci curves were produced on the 6th fully open leaf. N= 4, whilst error bars indicate the S.E. Results are the mean ± SE (n=4).Tkox-1 plants grown under (- -) conditions were not analysed.

3.6 DOXP supplementation can also complement the growth and chlorotic phenotypes.

DXS is a TPP dependent enzyme that is important not only for the MEP pathway but also for the biosynthesis of the thiazole moiety of thiamine (Julliard and Douce, 1991a). DXS is able to be bypassed if DOXP is supplied in the growth media (Julliard, 1992)whilst DXS mutants have been shown to have chlorotic phenotype (Araki et al., 2000). Because of this, supplementation of Tkox plants with DOXP was proposed. It was found that DOXP supplementation was able to complement the phenotype in seedlings but not to the extent of thiamine supplementation (Figure.3.14). Supplementation of TKox lines with pyruvate and G3P which are substrates used by DXS to produce DOXP were unable to complement the phenotype (Figure.3.15).

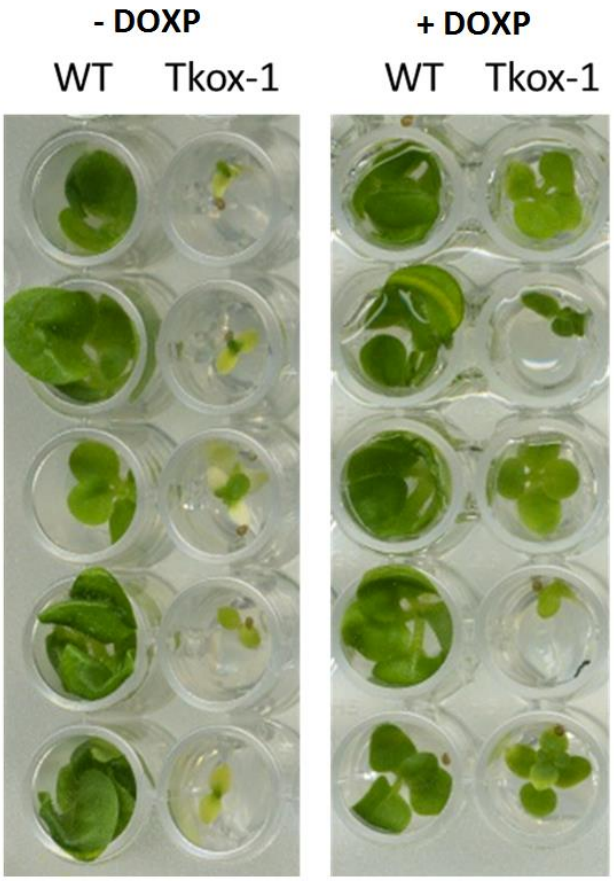

Figure 3.14- Deoxy-xylulose-5-phosphate (DOXP) supplementation can partially complement the chlorotic phenotype of TKox plants. Seedlings were germinated in MS media with and without additional DOXP (2mM). The growth of the seedlings was recorded 13 days after germination.

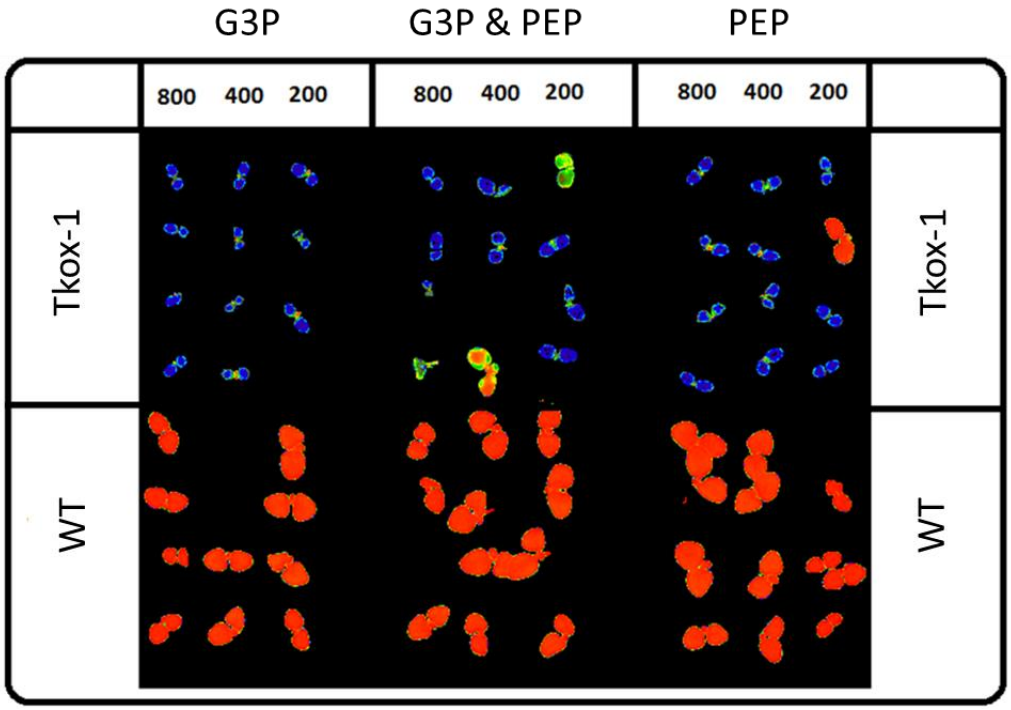

Figure 3.15-Supplementation with glyceraldehyde-3-phosphate (G3P) and/or phosphoenolpyruvic acid (PEP) does not complement the growth and chlorotic phenotypes in TKox lines. WT and TKox-1 lines supplemented with glyceraldehyde-3-phosphate and phosphoenolpyruvate. Seedlings were germinated in MS media with and without additional glyceraldehyde-3-phosphate (G3P) and phosphoenolpyruvic acid (PEP) at concentrations of 200, 400 and 800 µM and imaged using chlorophyll fluorescence (fv'/fm') to aid comparison.

3.7 Thiamine supplementation causes an increase in pre-dawn starch levels.

Starch levels in TKox lines have previously been shown to be increased at pre-dawn in comparison to WT plants (Khozaei, 2010) whilst mutations in the ThiC riboswitch which regulates the synthesis of the pyrimidine moiety of thiamine have also been shown to have an effect on starch metabolism (Bocobza et al., 2013). Therefore, a starch analysis was conducted on both WT and TKox lines both with and without an exogenous thiamine source in long (Figure.3.16) and short (Figure.3.17) day conditions.

It was found that supplementation of Tkox plants with thiamine in both long and short days caused an overall increase in starch levels in shoot tissue collected at pre-dawn. Long day plants were found to have higher starch levels than comparative short day plants. However, differences in the levels of starch between WT and TKox lines grown under the same conditions were negligible.

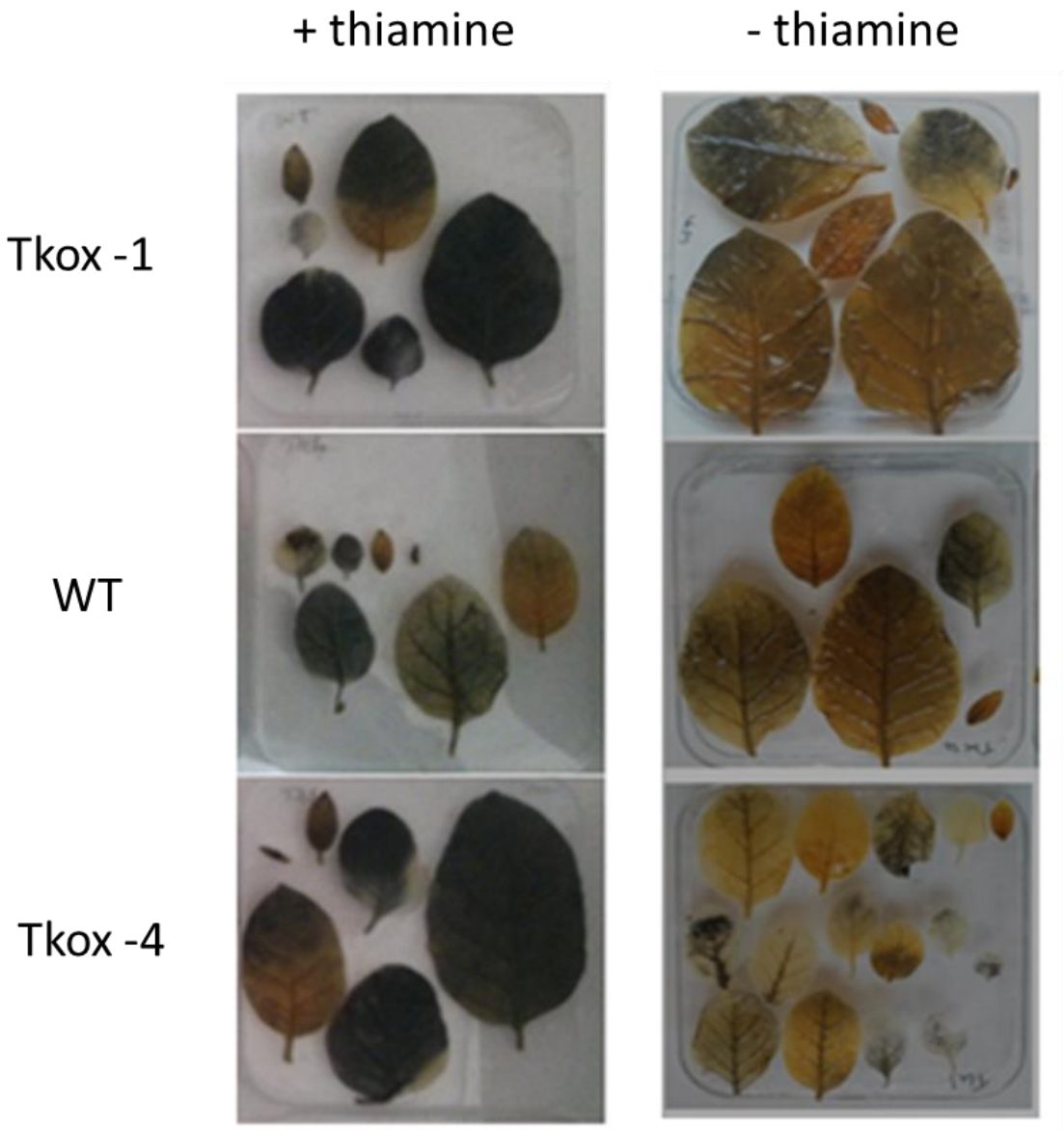

Figure 3.16- The effect of thiamine supplementation on starch accumulation in plants grown in long day conditions. Seeds from WT and Tkox lines were grown in long day conditions. One set of plants was supplemented with thiamine (50 mg L^{-1}) whilst the second set was not. At 3 weeks post germination, leaf material was analysed pre-dawn for its starch content.

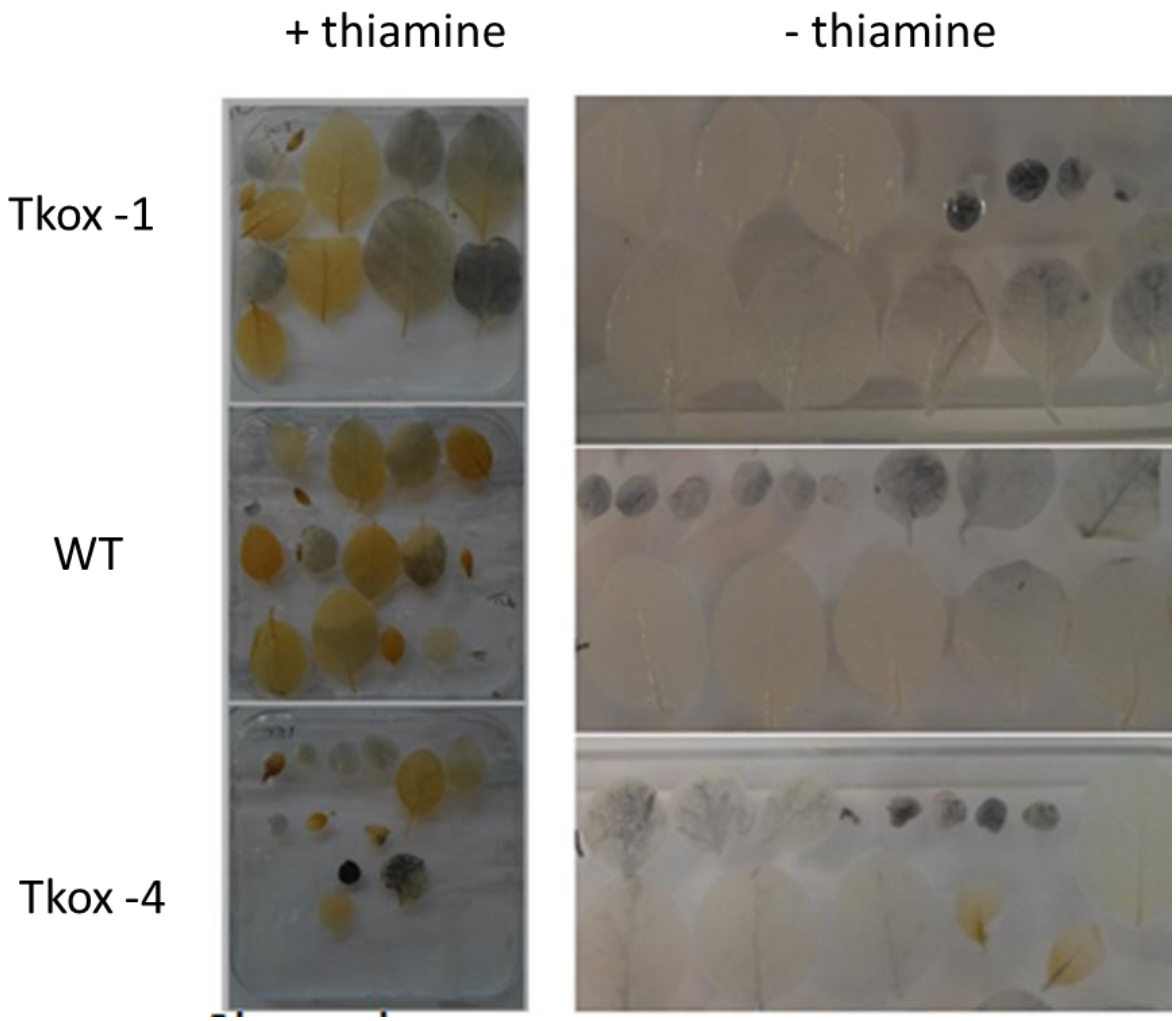

Figure 3.17- The effect of thiamine supplementation on starch accumulation plants grown in short day conditions. Seeds from WT and Tkox lines were grown in short conditions. One set of plants was supplemented with thiamine (+ thiamine) at 50 mg L^{-1} whilst the second set was not (- thiamine). At 3 weeks post germination, leaf material was analysed pre-dawn for its starch content.

Discussion

Three TKox lines from the T3 generation of transketolase over-expressing lines were analysed for the effect of Increased TK activity and thiamine supplementation on their biochemical and physiological properties. This chapter has shown that the TKox lines analysed had increases in total TK protein levels of between 2 and 2.5 that of WT plants. This increase caused a significant decrease in the growth of both the root and shoot system and also a chlorotic phenotype. In addition, the Tkox -1 and -8 lines which showed the highest increase in total TK protein also had the most severe phenotypes.

Previous studies of these TKox lines had shown that increased plastid TK expression resulted in a partial thiamine auxotrophic phenotype that was able to be rescued with the availability of either thiamine or TPP in the germination media (Khozaei, 2010). However, the finding that imbibing seeds in a thiamine solution was also able to recover the growth phenotype in TKox lines indicates that the growth phenotype in particular is attributed to insufficient thiamine levels contained within the seed.

These results have also shown that the timing of thiamine application is crucial in the recovery of the growth and chlorotic phenotype. Thiamine supplementation at the point of germination is essential to recover the growth phenotype whilst the chlorotic phenotype can be rescued at any time with the latter re-appearing if thiamine supplementation is withdrawn.

The ability to recover the growth phenotype if thiamine levels were increased in the seed either through imbibition or germination of seeds on a thiamine enriched media may be due to an impairment of normal meristem development. Analysis of a maize thiamine mutant showed that the development and maintenance of the shoot apical

meristem is sensitive to thiamine deprivation which leads to the premature abortion of shoot apical meristems (Woodward et al., 2010). Mutants deficient in thiamine biosynthesis have also been shown to have reduced root growth (Zhao et al., 2011) indicating that thiamine deficiency may also inhibit root meristem development which would correspond with these results which show that thiamine/TPP supplementation post 12 days germination failed to recover the growth phenotype.

In contrast, the ability to recover the chlorotic phenotype at any period of development in Tkox lines indicates that chlorophyll biosynthesis is interrupted unless a continuous exogenous thiamine supply is available. This finding is in agreement with studies of Arabidopsis thiamine mutants which showed that the chlorotic phenotype could be complemented when the plants were supplied with an exogenous thiamine source (Li and Redei, 1969) as were plants with a mutation in the production of chlorophyll pigment biosynthesises which results from thiamine deficiency in the chloroplasts (McHale et al., 1988).

One explanation for this can be attributed to the fact that both thiamine and chlorophyll biosynthesis pathways share a common precursor in deoxy xylulose phosphate (DOXP) which originates from the condensation of G3P and pyruvate by the enzyme 1-deoxy-d-xylulose-5-phosphate synthase (DXS). DXS is a TPP dependent enzyme that is important for the biosynthesis of the thiazole moiety of thiamine. DXS has also been shown to be a rate limiting step in the DOXP/MEP pathway(Estévez et al., 2001) and is regulated by a number of factors including both the transcript and protein levels of the DXS enzyme (Guevara-Garcia et al., 2005), light (Loivamaki et al., 2007) as well as MEP pathway intermediates that compete with TPP for binding to DXS (Banerjee et al., 2013).

However, it should be taken into consideration that eukaryotes are currently thought to only use glycine and NAD+ in the production of the thiazole moiety (Chatterjee et al., 2007) whilst bacteria use DOXP for the biosynthesis of thiazole (Kronenberger et al., 2013). However there is evidence also to show that plants are also able to utilize DOXP as in bacteria (Julliard and Douce, 1991a).

Further evidence of the role of DXS in this phenotype is provided by both the increase in DXS protein levels as well as the finding that DOXP supplementation is able to partially complement the growth and chlorotic phenotype in Tkox seedlings. DOXP supplementation has been shown to be able to bypass the DXS enzyme (Julliard and Douce, 1991a). This indicates that decreased production of the thiazole moiety may be the cause of the growth and chlorotic phenotype as has been shown in a temperature sensitive DXS mutant that also exhibited a chlorotic phenotype in young tissues (Araki et al., 2000).

The explanation as to why DOXP partially recovers the phenotype would require further investigation especially as supplementation with its substrates: G3P and pyruvate failed to have any effect. It may be that TPP levels are limiting the activity level of the DXS protein and thereby affecting the balance of carbon flow to between the thiamine biosynthesis pathway as well as the isoprenoid and starch pathways.

Photosynthetic carbon assimilation rates in TKox lines had previously been found not to significantly differ if the chlorotic part of the leaf was avoided when taking measurements (Khozaei, 2010). However, it was unknown what the effect of thiamine supplementation would have on carbon assimilation and whether thiamine supplemented Tkox lines would have higher rates of carbon assimilation than that of WT plants. As expected, the falls in assimilation rates were most severe in those

plants that were not supplemented with thiamine and therefore exhibited the chlorotic phenotype. This finding is not unexpected as TK is a C_3 cycle enzyme that has been identified as exerting significant control over RuBP regeneration (Harrison et al., 1998; Zhu et al., 2007) (Henkes et al., 2001).The analysis of the assimilation rates of the Tkox plants supplemented with thiamine showed that increased assimilation was evident over non-thiamine supplemented plants. Despite this, these results have demonstrated that increasing TK activity in tobacco, even with supplemental thiamine, is unable to increase photosynthetic rate and growth above that of WT plants.

The function of TK is pivotal in carbohydrate metabolism as erythrose-4-phosphate is a known inhibitor of phosphoglucose isomerase (PGI) (Grazi et al., 1960) which converts fructose-6-phosphate (F6P) to glucose-6-phosphate (G6P) in the 4^{th} enzymatic reaction of both sucrose and starch biosynthesis which occur in the cytosol and chloroplast respectively.

Previous research had shown TKox lines showed an accumulation of starch in pre-dawn plants that were grown in long and short day conditions which was attributed to the reduced growth phenotype causing sugars to accumulate (Khozaei, 2010). In contrast, TK antisense plants failed to demonstrate any changes to starch levels but did have an effect on sucrose levels (Henkes et al., 2001)

Analysis of starch levels in both WT and TKox lines that were supplemented with an exogenous thiamine source showed that thiamine supplementation led to increased starch accumulation in both WT and TKox lines. Whilst the differences in starch levels between WT and Tkox plants were negligible when grown under the same conditions. The reason for this result is unknown but it may be attributed to the sensitivity of Lugol's starch staining method. Despite this, there was a clear increase in the starch

levels of plants that were supplemented with thiamine during development in both long and short day conditions. A similar result was seen in Arabidopsis plants with a mutated ThiC riboswitch that accumulated excess thiamine reserves (Bocobza et al., 2013). This finding suggests it is changes to thiamine levels rather than transketolase that have an effect on starch metabolism.

This chapter has shown that TKox lines have an increase in total TK protein levels of between 2 and 2.5 times that of comparative WT plants. The effect of this increase is a growth and chlorotic phenotype that can be rescued with thiamine. However, the growth phenotype can only be rescued if thiamine is supplied at the point of germination which suggests that insufficient levels of thiamine contained within the seeds. In addition to the increase seen in TK protein levels, levels of DXS were also shown to be increased.

The supplementation of Tkox seedlings with DOXP, the product of the DXS enzyme was able to partially complement the phenotype and has indicated that the cause of the phenotype in these plants may be attributed to a reduction in flux through the DOXP/MEP pathway leading to insufficient levels of the thiazole moiety of thiamine being synthesized.

CHAPTER 4

Thiamine metabolism of TKox lines

Introduction

Thiamine (vitamin B1) is a precursor to thiamine pyrophosphate (TPP) which is a co-factor for enzymes that are involved in the Calvin cycle, pentose phosphate pathway, glycolysis, the TCA cycle and branched chain amino acid metabolism. Because thiamine availability is essential for these processes to function it is therefore vital for plant viability.

The synthesis of thiamine occurs in prokaryotes, fungi and plants whilst mammals must obtain thiamine through their diet. In plants thiamine biosynthesis takes place in the chloroplast (Belanger et al., 1995) where two independently produced pyrimidine (HMP-PP) and thiazole moieties (HET-P) are condensed to form thiamine mono-phosphate (TMP). TMP is then dephosphorylated and exported into the cytosol where it is then phosphorylated first to TMP and then thiamine pyrophosphate (TPP) by the enzyme thiamine phosphokinase (TPK).When thiamine is in its active TPP form it can then be transported back into the chloroplast to act as a co-factor for: transketolase (TK), pyruvate dehydrogenase (PDH), deoxy-xlylulose synthase (DXS) and acetolactate synthase (ALS). Import of TPP into the mitochondria allows the function of the TPP dependent enzymes: pyruvate dehydrogenase (PDH), pyruvate decarboxylase (PDC), ketoglutarate dehydrogenase (KGDH) and branched chain ketoacid dehydrogenase (BCKDH).

Production of the thiazole moiety of thiamine commences with the condensation of glyceraldehyde-3-phosphate (G3P) and pyruvate by the enzyme deoxy-xylulose synthase (DXS) which forms 1-deoxy-D-xylulose 5-phosphate (DOXP) (Julliard, 1992). Alternatively it has also been shown that glycine and NAD+ are used in the first step thiazole biosynthesis (Godoi et al., 2006).In the next step the enzyme thiamine

thiazole synthase (Thi1) produces 5-(2-hydroxyethyl)-4-methylthiazole phosphate (HET-P).

In contrast the synthesis of the pyrimidine moiety of thiamine has two steps involved in its production. In the first step, HMP-P synthase (ThiC) converts 5-amino imidazole ribonucleotide (AIR) to 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate (HMP-P) which requires S-adenosyl-methionine (S-AM) and NADH. In the next reaction, the multi-function enzyme hydroxymethyl pyrimidine kinase (TH1) phosphorylates 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate (HMP-P) to 4-amino-2-methyl-5-hydroxymethylpyrimidine di-phosphate (HMP-PP). The TH1 enzyme then carries out its second role by joining both the thiazole and pyrimidine moieties to form thiamine monophosphate.

The synthesis of thiamine is a highly regulated process that has been shown to be co-ordinated by a metabolite sensing mRNA mechanism that is thought to have pre-dated the emergence of proteins (Winkler et al., 2002). This mechanism is known as the ThiC riboswitch because it regulates the production of the ThiC protein which is vital for the production and regulation of the pyrimidine moiety of thiamine. This occurs through the alternative splicing of nascent ThiC mRNA which is dependent on the levels of TPP. When TPP binds a sequence in the 3'UTR in land plants this causes the nascent mRNA to fold which exposes specific sequences that are then spliced out of the original mRNA sequence (Raschke et al., 2007; Wachter et al., 2007; Kong et al., 2008).The effect of this is degradation of the mRNA structure leading to a fall in ThiC protein levels and blockage of production of the pyrimidine moiety (Wachter et al., 2007). It has recently been found that ThiC levels are also regulated by the circadian clock where results showed that the THIC promoter directs gene expression in a time specific manner that is not sensitive to TPP levels (Bocobza et al., 2013).

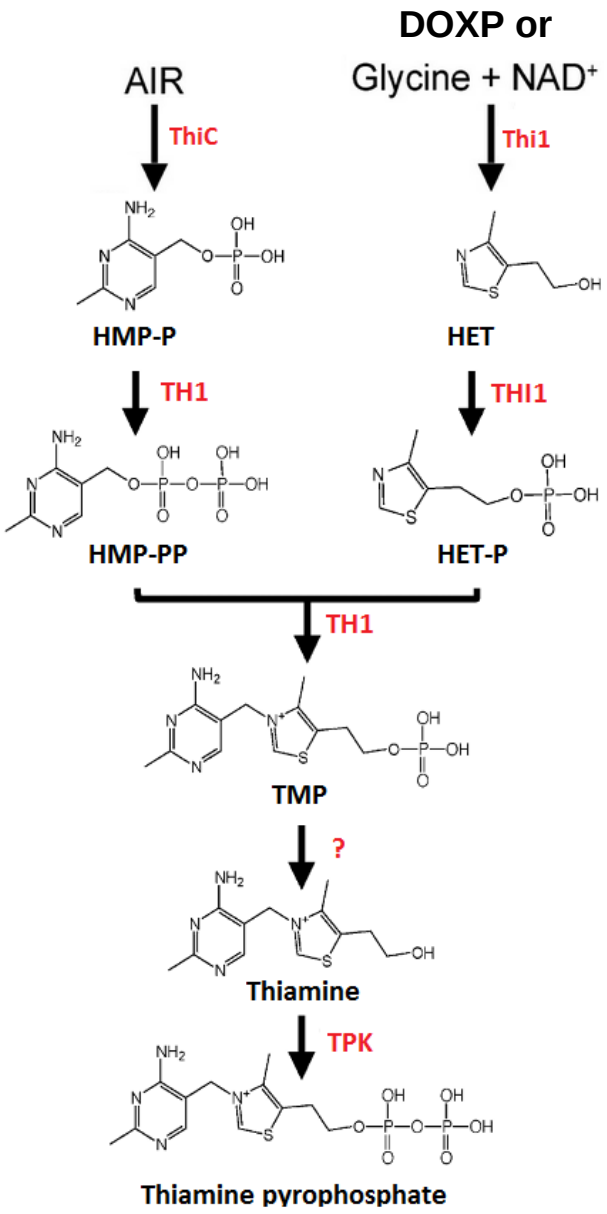

Figure 4.1- The biosynthesis of thiamine. Thiamine is created from the condensation of pyrimidine (HMP-PP) and thiazole (HET-P) moieties by the enzyme TH1 to form thiamine monophosphate (TMP). TMP is then dephosphorylated to thiamine and transported across the chloroplast envelope into the cytosol where it is phosphorylated to TPP by thiamine phosphokinase (TPK). It can then be transported into both the chloroplast and mitochondria to be used as a co-factor by TPP dependent enzymes.

Though ThiC is considered the main regulatory mechanism controlling thiamine biosynthesis in higher plants, there are also regulatory mechanisms involved in the production of intermediates in the thiazole pathway. DXS which produces DOXP for thiazole biosynthesis has been identified as being a highly regulated enzyme.

The activity levels of the DXS enzyme have been shown to be effected by the quantity of both *DXS* transcripts and protein present (Guevara-Garcia et al., 2005) whilst both isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP), which are products of the MEP have been shown to compete with TPP for access to the co-factor binding site (Banerjee et al., 2013).

The previous chapter has demonstrated that the growth and chlorotic phenotype caused by increases in TK protein can be rescued with an exogenous thiamine source though the timing of this application is critical in allowing recovery of these phenotypes which were shown to be independent of each other. Therefore this chapter will investigate how the levels of both thiamine and TPP are altered throughout the lifecycle of Tkox plants. In addition, both qPCR and RNA-seq analysis will be utilised to try and establish the mechanism linking increased levels of TK protein with thiamine metabolism.

Results

4.1 Seed thiamine levels.

To identify variations in the levels of thiamine, seeds from both WT and the two most severely affected TKox lines (-1 and -8) were imbibed in either water or thiamine (10Mg L^{-1}). In addition, seeds from plants that received thiamine supplementation throughout their lifecycle were also analysed for their thiamine content (Figure.4.2). The results showed that the thiamine content of the dry seeds from both Tkox -1 and -8 lines was less than half that of the comparable WT seeds. However, by imbibing the seeds in a thiamine solution, the levels of thiamine contained within the seed were found to be similar to that in WT seeds. In addition, supplying Tkox plants with an exogenous thiamine source throughout the life cycle resulted in the seeds produced from these plants having similar thiamine levels to WT. This increase in thiamine levels was up to 2.5 fold higher than that of the seed from plants that received no thiamine supplementation

To test whether the increase seen in seed thiamine levels by imbibing seeds from TKox lines in thiamine was attributed to the process of thiamine binding to thiamine binding proteins or just being retained by the seed coat, seeds from the same seed batch as used in figure 4.2 were imbibed in a TPP solution (10Mg L^{-1}) (Figure.4.3). TPP levels in the seeds were negligible (not shown) whilst thiamine levels in TKox lines were shown to be increased by up to 30% compared to dry seed. Further testing identified the increase in the thiamine levels of TPP imbibed seeds to be due to the breakdown of TPP to thiamine monophosphate (TMP) and thiamine in water but not in a 10% trichloroacetic acid (TCAA) solution (Figure.4.4).

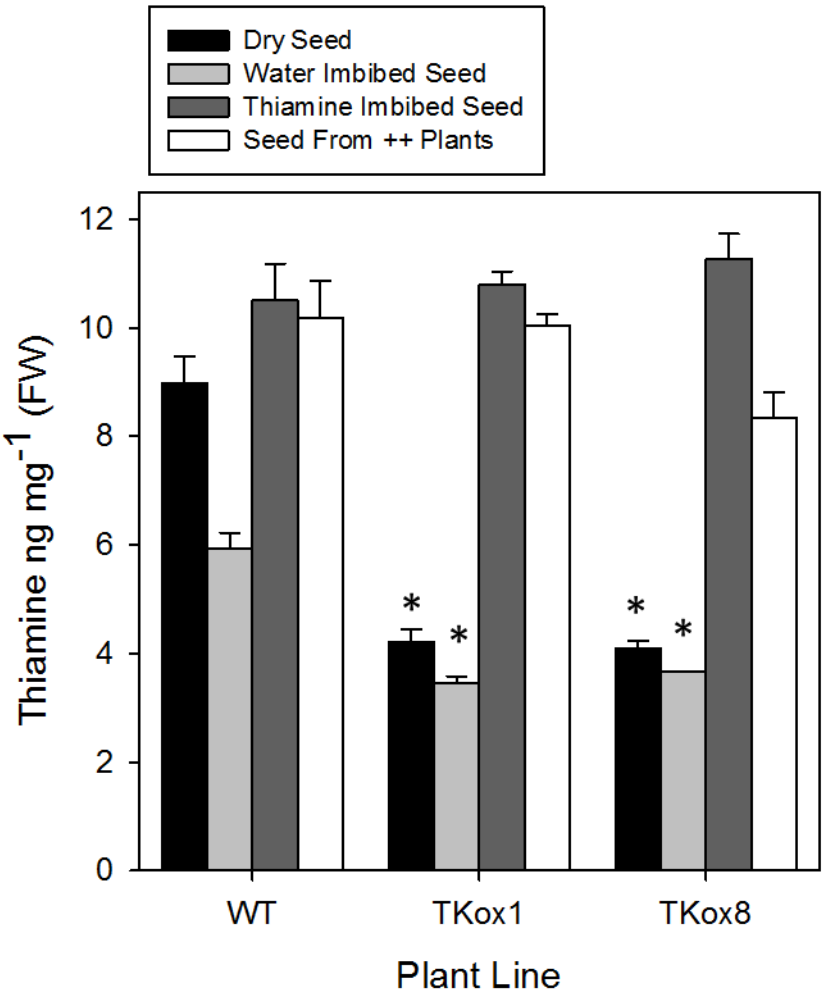

Figure 4.2- Thiamine levels in TKox and WT seeds. Seeds were analysed for thiamine content when dry or following 24 h in thiamine (10 mg L^{-1}). The parental ++ plants were watered with thiamine (50 mg L^{-1}) throughout their lifecycle. Results are the mean ± SE (n = 4). Statistical comparison was made using a one way Anova (95 % sig) and Tukey post hoc test. * indicates significance at $P< 0.05$.

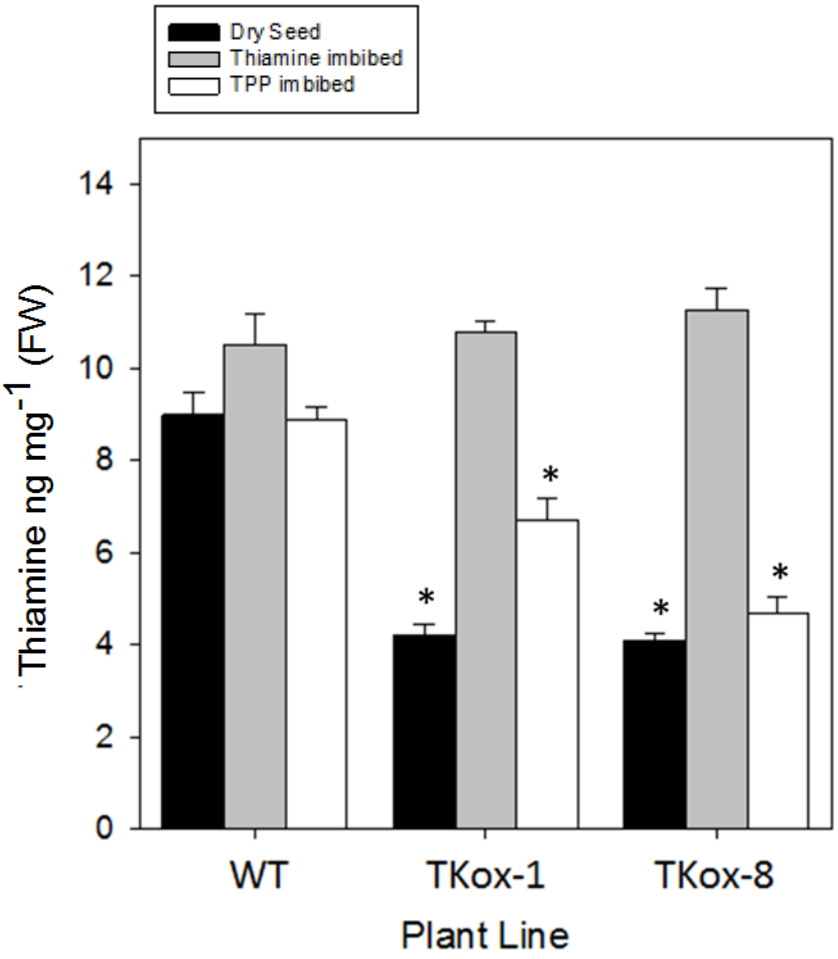

Figure 4.3- Thiamine content of seeds after imbibing in TPP solution. Seeds were analysed for thiamine content after being imbibed in TPP (10 mg L^{-1}) for 24 hours. Results are the mean ± SE (n=4). Statistical comparison was made using a one way Anova (95 % sig) and Tukey post hoc test. * indicates significance at P< 0.05.

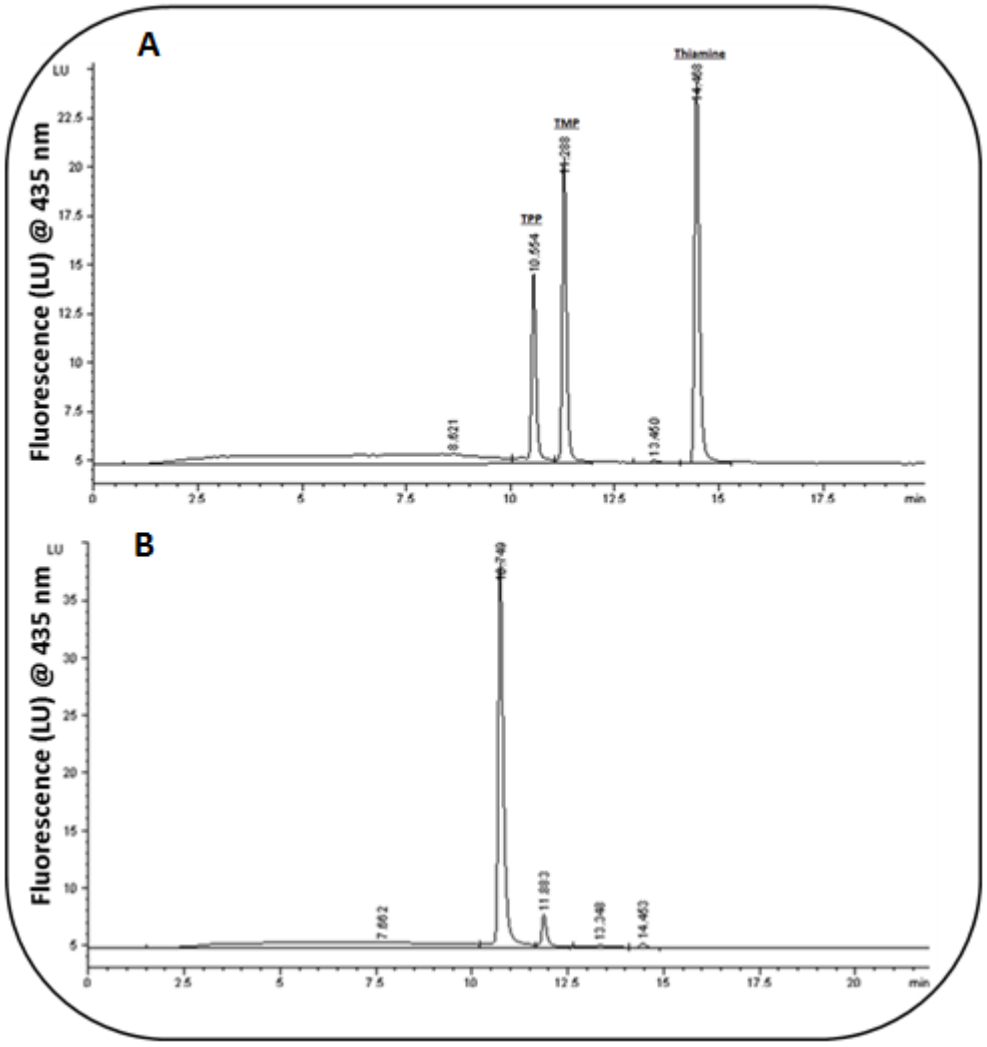

Figure 4.4- Stability of TPP in acidic and neutral solution. TPP was resuspended at a concentration of 800 nmol in both 10% trichloroacetic acid (A) and H_2O (B) to ascertain its stability in these conditions. Three independent peaks were identified by comparing against a known standard. Elution times were: TPP (~10.5 mins), TMP (~11.2 mins) and thiamine (~14.5 mins).

4.2 Thiamine but not TPP levels are reduced in Tkox seedlings.

To explore the consequences of reduced thiamine in the seeds of the Tkox plants on TPP availability during seedling establishment, shoot tissue was taken from young seedlings at 3, 6, 9 and 12 days post germination and analysed via HPLC (Figure.4.5).Thiamine levels in all of the TKox lines were found to be significantly lower than that seen in WT plants between 3-9 days post germination. In contrast, the levels of TPP in TKox lines were not significantly different from WT, with the exception of the 3 day old Tkox -1 seedlings. At 12 days post germination there was a uniform increase in both thiamine and TPP levels with the mean values of thiamine and TPP found not to significantly differ from one another in WT and TKox lines.

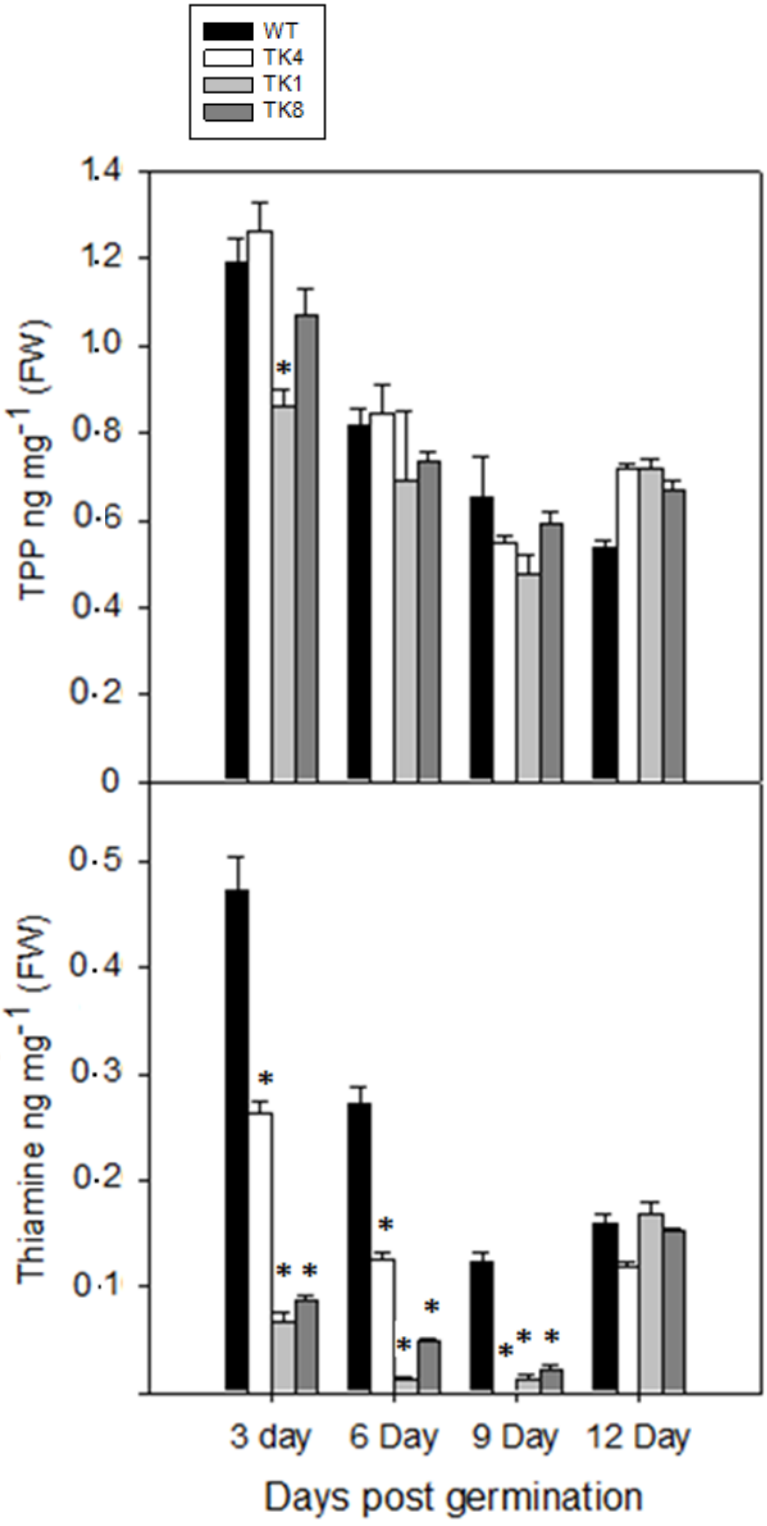

Figure 4.5- Thiamine and TPP levels in seedlings. TKox lines -1, -4, -8 and WT plants were grown in MS media and shoot material was taken at 3, 6, 9 and 12 days post germination and analysed via HPLC for thiamine and TPP levels. Results are the mean ± SE (n = 4). Statistical comparison was made using a one way Anova (95 % sig) and Tukey post hoc test. * indicates significance at P< 0.05.

4.3 Thiamine supplementation increases thiamine and TPP levels.

It was established in the previous chapter that the application and withdrawal of thiamine supplementation at specific stages of development has a direct effect of the recovery of both the growth and chlorotic phenotypes seen in TKox lines. The growth phenotype was only fully recovered when thiamine supplementation was given at the point of germination. In contrast, the chlorotic phenotype was recovered regardless of the timing of thiamine supplementation. However, if thiamine is subsequently withdrawn, the chlorotic phenotype was found to re-appear in the leaves.

To investigate how thiamine and TPP levels differed in WT and TKox lines under different thiamine supplementation regimes, plants were germinated in media containing thiamine (10 Mg L^{-1}) and then transferred to soil at 12 days post germination and watered with (++) or without (+-) thiamine. A second set of plants were germinated without thiamine and watered with (-+) or without (--) thiamine.

In the rosettes of WT and TKox lines -4, -1 and -8 at 21 days post germination it was shown that thiamine supplementation resulted in an increase in thiamine and TPP levels of between 5 to 8 fold compared to non-supplemented plants (Figure 4.6). However, in ++ supplemented conditions, WT plants accumulated ~50% more TPP and thiamine than comparative Tkox in the majority of cases. In contrast, plants under the -+ supplementation regime showed the opposite with TKox lines having increases of ~50% of both thiamine and TPP levels above that seen in WT plants. Both +- and -- supplementation regimes showed that there was no significant difference between thiamine and TPP levels under these conditions. The levels of thiamine and TPP differ if sampled from developmental stage rather than on a time based sampling method;

tissue from the 6th fully open leaf was analyzed for both thiamine and TPP levels under the same thiamine supplemental regimes used in the rosettes (Figure.4.7)

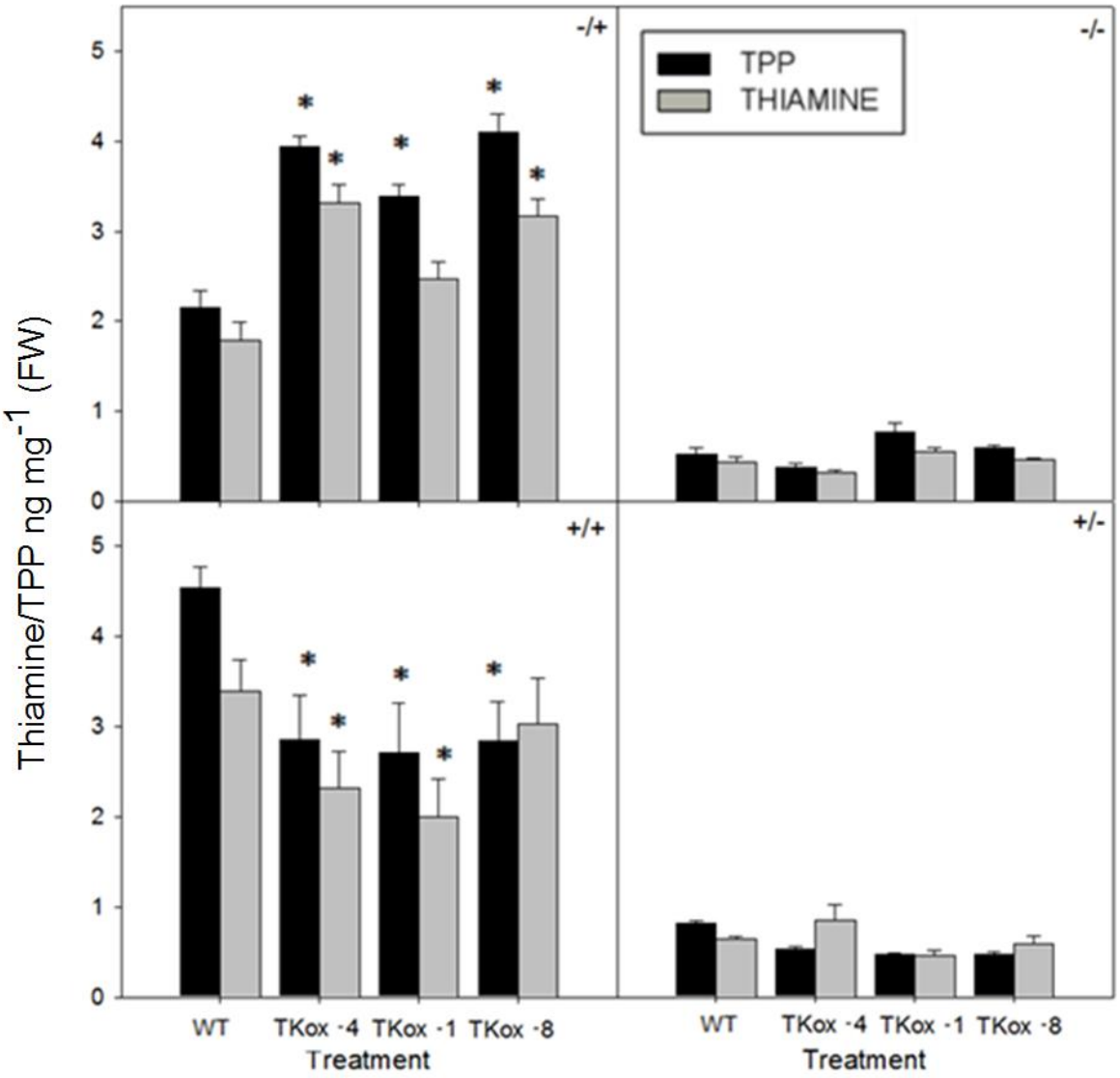

Figure 4.6- Thiamine and TPP levels in 21 day old rosettes of WT and Tkox lines under a varied thiamine supplementation regime. TKox and WT plants were germinated in media containing thiamine (50 mg L^{-1}) and then transferred to soil 12 days after germination (DAG) and watered with thiamine (+/+) or without (+/-). A second set of plants were germinated without thiamine and watered with thiamine (-/+) or without (-/-). Plants were transferred to soil 12 DAG. Results are the mean ± SE (n = 5). Statistical comparison was made using a one way Anova (95 % sig) and Tukey post hoc test. * indicates significance at P< 0.05.

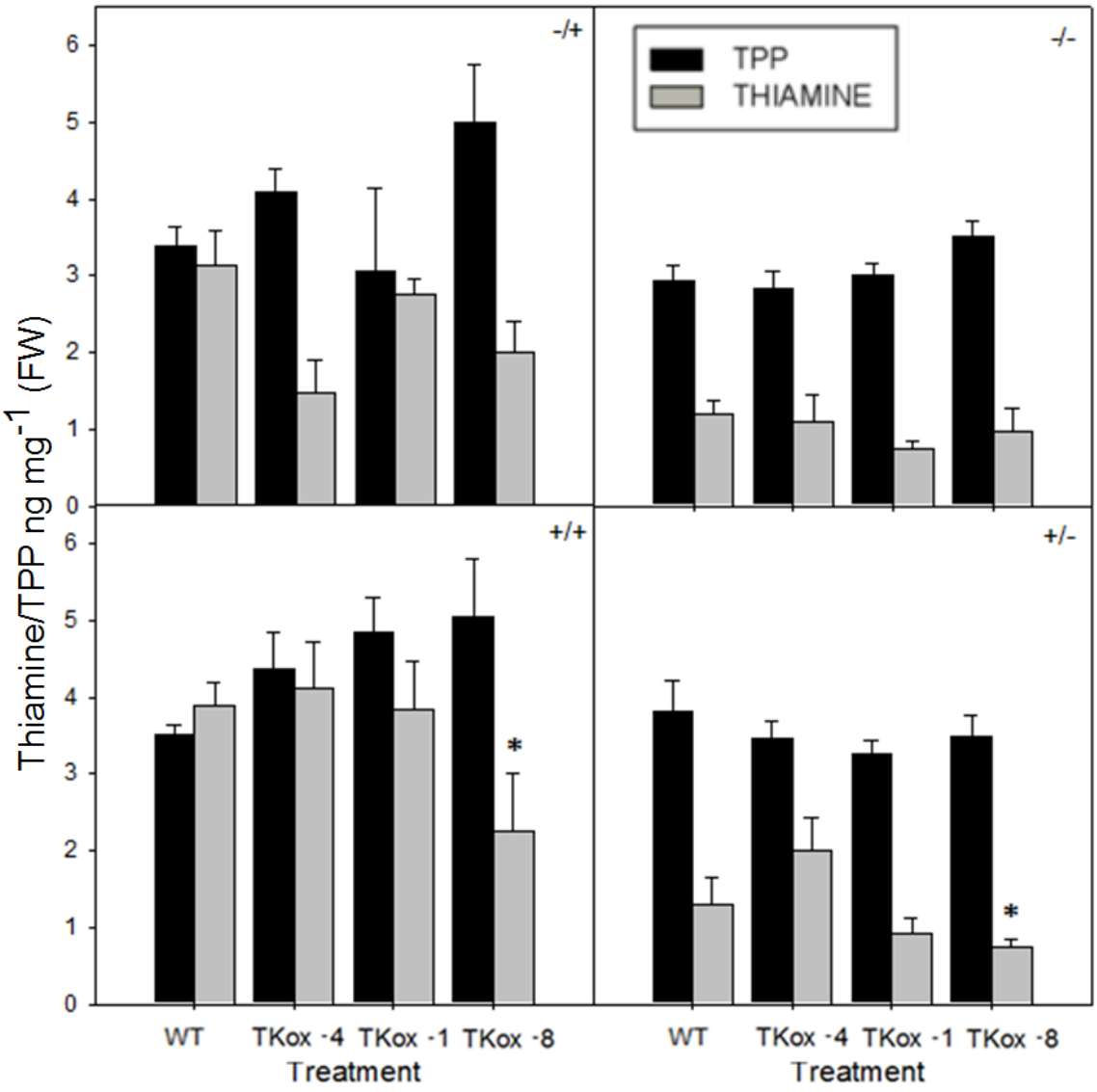

Figure 4.7- Thiamine and TPP levels in the 6th fully open leaf under a varied thiamine supplementation regime. TKox and WT plants were germinated in media containing thiamine (50 mg L^{-1}) and then transferred to soil 12 days post germination and watered with thiamine (++) or without (+-). A second set of plants were germinated without thiamine and watered with thiamine (-+) or without (--). Leaf tissue was removed and processed via HPLC for tits thiamine and TPP content. Results are the mean ± SE (n=5). Statistical comparison was made using a one way Anova (95 % sig) and Tukey post hoc test. * indicates significance at $P < 0.05$.

The results showed that supplemental thiamine causes an increase in the level of both thiamine and TPP levels as was found in the rosette material. However, in the 6th leaf, TKox lines under the ++ and -+ thiamine supplementation regimes had levels of TPP that were slightly higher than comparable WT plants but this was not statistically significant in the majority of cases. In comparison, – and +- watering regimes, thiamine and TPP levels also failed to show a significant difference to WT values except in the case of Tkox -8 line under +- supplementation.

4.4 Thiamine and TPP levels are reduced in the youngest leaves of flowering Tkox plants.

It was shown that in chapter.3 that insufficient thiamine levels in the seed of the Tkox plants is the cause of the growth and chlorotic phenotype in Tkox seedlings. To investigate the reasons for the decrease in thiamine in the TKox lines, an analysis of thiamine and TPP levels was conducted on the leaves of flowering WT and Tkox plants when they were at the same developmental stage. However, because the presence of the growth phenotype limits the developmental rate in TKox lines and therefore the developmental timing of floral organs it was deemed necessary to provide thiamine at the point of germination so that both WT and Tkox plants would have similar growth rates and therefore produce plants of a similar size and leaf number. Seeds of both WT and TKox lines were soaked in thiamine (10 Mg L^{-1}) for 24 hours before sowing. Plants were then grown until the first flower opened and the leaves sampled across the entire plant from leaf 8 (oldest remaining leaf) to leaf 21 (youngest fully expanded leaf). The levels of both thiamine and TPP were found to be highly variable in the lower leaves of the plant but the youngest leaf (leaf 21) showed significantly lower levels of both TPP and thiamine levels in the Tkox plants when compared to WT plants (Figure.4.8). However, when stem tissue from these same plants was analysed for thiamine and TPP content, only thiamine and not TPP levels were shown to significantly different between the WT and Tkox plants (Figure.4.9).

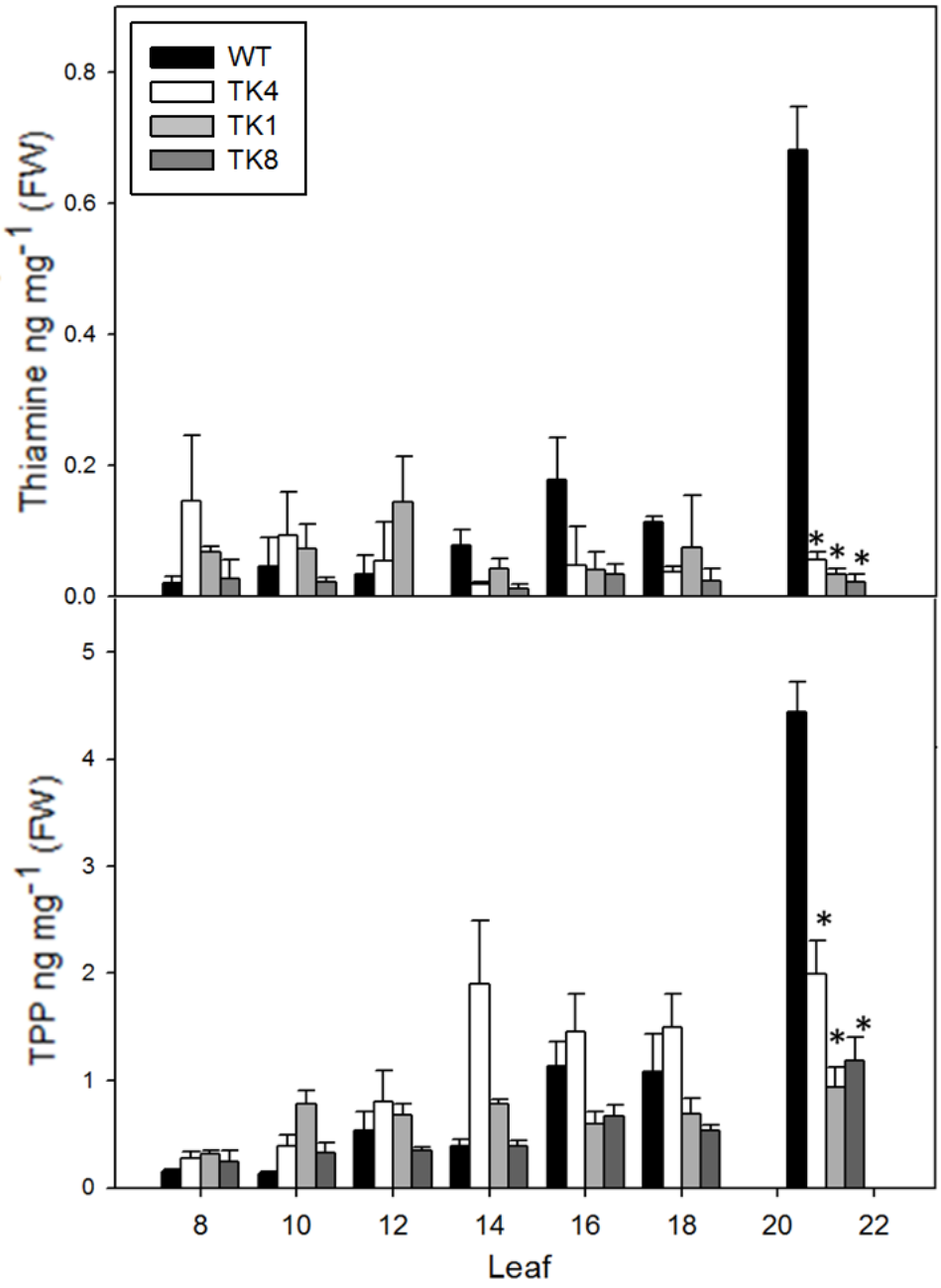

Figure 4.8- The levels of thiamine and TPP in the leaves of flowering plants. Seeds from WT and Tkox lines were imbibed in thiamine solution and then grown under long day conditions in the greenhouseunder light levels of between 600 to 1500 µmol m^{-2} s^{-1}, 25°C. Leaf samples were taken from: 8 (oldest), 10,12, 14, 16, 18, 20 and 22 (youngest) when the first flower opened. to measure thiamine/TPP levels. Results are the mean ± SE (n = 5). Statistical comparison was made using a one way Anova (95 % sig) and Tukey post hoc test. * indicates significance at $P < 0.05$.

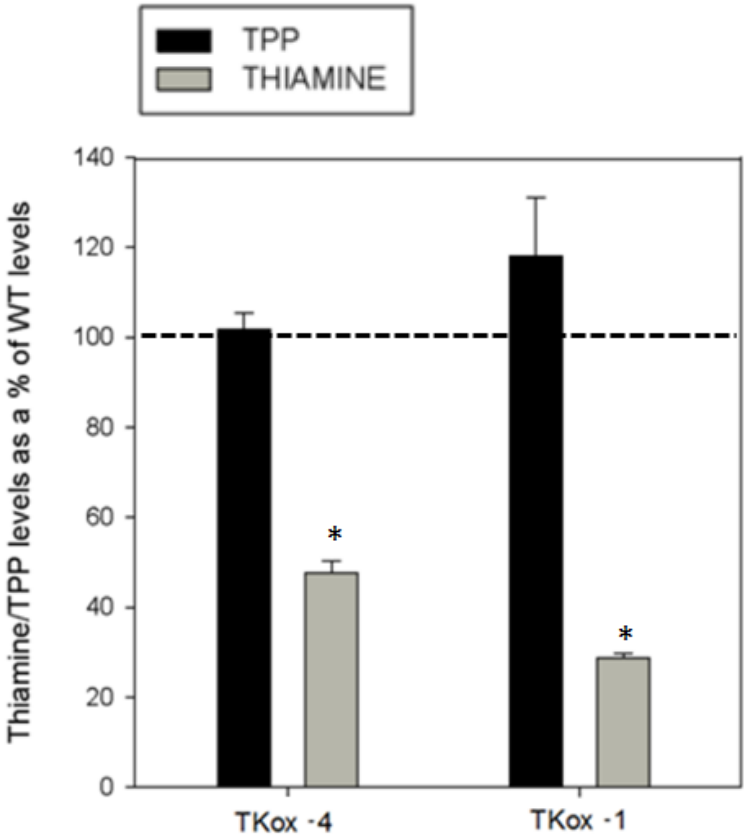

Figure 4.9- The levels of thiamine and TPP in the stems of flowering plants. Samples were taken from the upper, middle and lower stem of each plant. A pooled tissue sample was then analysed for thiamine and TPP levels via HPLC. Results are the mean ± SE (n=4). Statistical comparison was made using a one way Anova (95 % sig) and Tukey post hoc test. * indicates significance at $P < 0.05$.

4.5 ThiC transcript variants indicate a low TPP phenotype in TKox lines.

The 3'UTR of the ThiC nascent mRNA which encodes the enzyme thiamine synthase C contains a TPP regulated riboswitch (Figure.4.10). Unbound TPP is able to bind the 3' UTR region of ThiC mRNA which, dependent of the level of TPP available can alter translation of the ThiC mRNA and subsequently the biosynthesis of the pyrimidine moiety. When the levels of TPP are high free unbound TPP can bind to the 3' UTR of ThiC mRNA causing an alternative splice event which leads to an increase in the levels of the ThiC3 variant. When TPP levels are low then ThiC2 is the dominant splice variant whilst ThiC variant 1 indicates presence of the coding sequence.

In all three TKox lines the abundance of the ThiC2 splice variant was found to be higher than WT plants, whilst the quantity of ThiC 3 transcripts was found to be lower which is typical of low TPP phenotype (Figure.4.11). Thiamine supplementation was found to increase the levels of ThiC3 transcripts in TKox lines indicating that sufficient TPP levels are available in these plants (Figure.4.12). However, the number of ThiC1 and ThiC2 transcripts still remained higher than comparative transcripts in WT plants.

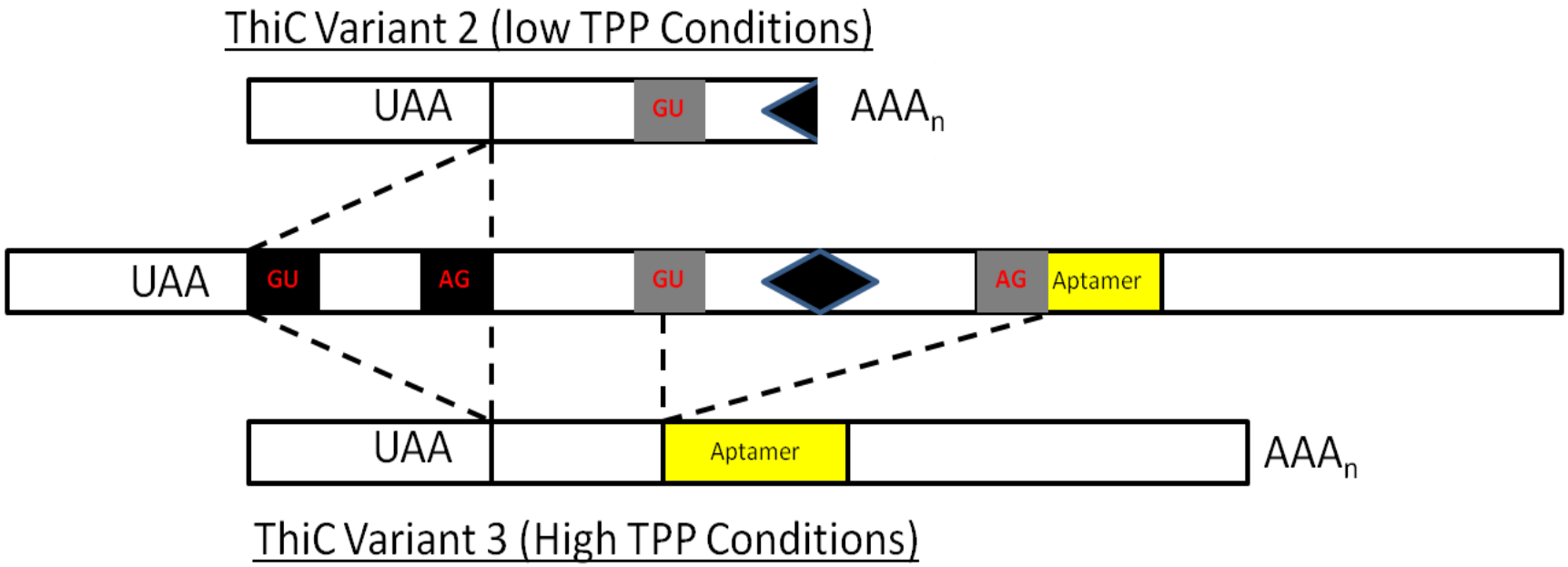

Figure 4.10 Alternative mRNA processing of ThiC UTR via the TPP riboswitch mechanism (redrawn from (Wachter et al., 2007). UAA identifies the stop codon of the open reading frame. GU and AG identify the site of the first intron which is spliced from both ThiC2 and ThiC3 variants. The diamond identifies the ThiC splice 2 variant utr termination site whilst GU and AG identify the site of the second splice site used to produce ThiC splice 3 variants which are present in high TPP conditions. The aptamer site in yellow is the TPP binding site.

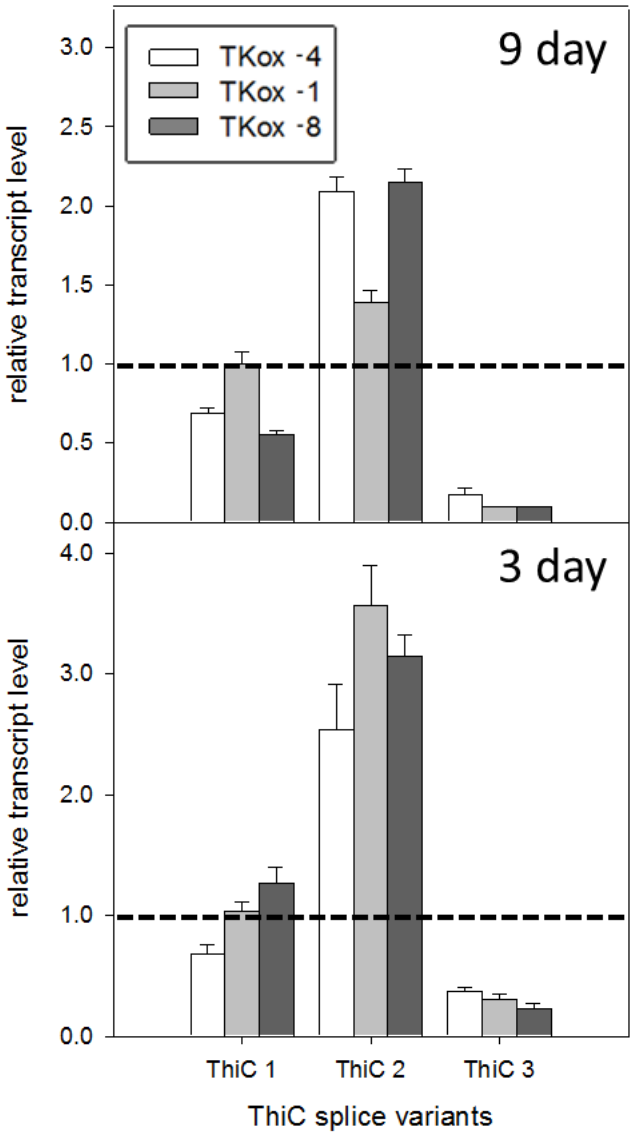

Figure 4.11- qPCR analysis of transcript levels of *ThiC* gene. Splice variant transcripts of the ThiC gene (ThiC1, ThiC2 and ThiC3) were determined by qPCR, the dashed line indicates WT. The values represent a pooled sample of whole plant material taken at 3 and 9 days post germination.

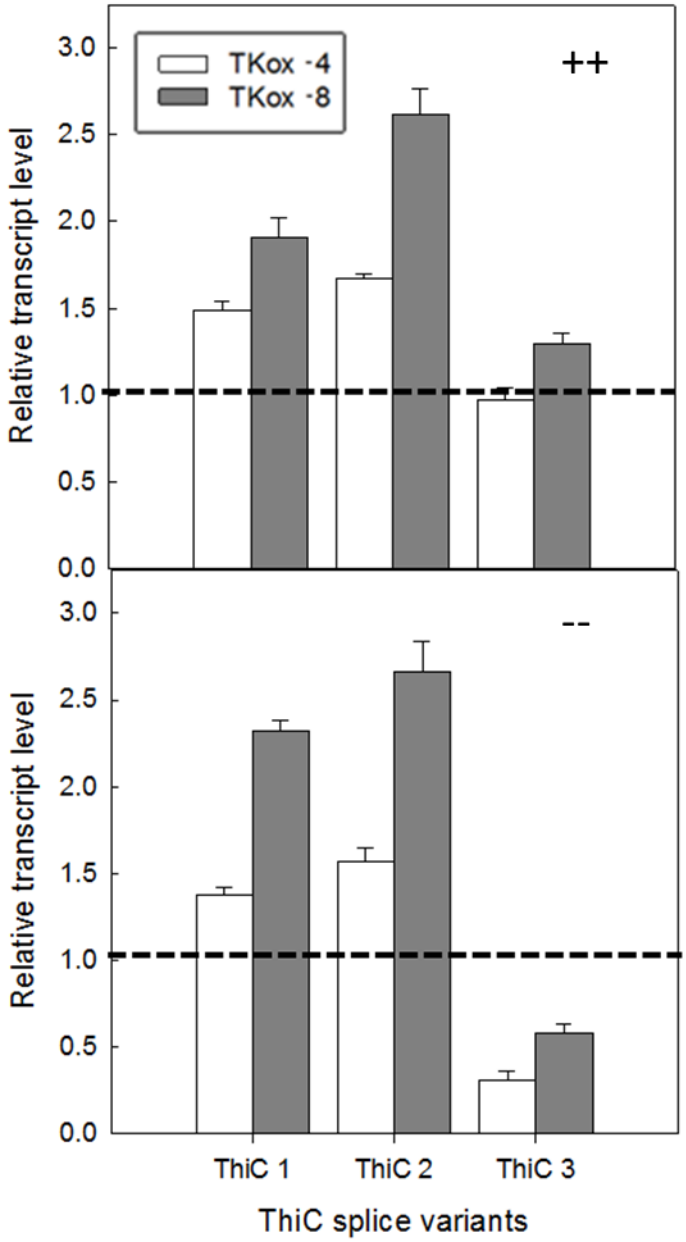

Figure 4.12 ThiC transcript levels in thiamine supplemented/non-supplemented Tkox lines. Relative levels of the splice variant transcripts of the ThiC gene (ThiC1, ThiC2 and ThiC3) were determined by qPCR, the dashed line indicates WT. The values represent a pooled sample of whole plant material taken from the total shoot system of 21 day old plants that were given constant thiamine supplementation (++) and plants that received no thiamine supplementation (--). N = pooled sample of RNA from 3 separate plants.

4.6 The transcript levels of TPP dependent genes in TKox lines.

Because the analysis of the ThiC transcripts indicated a TPP deficient phenotype 3 and 9 day post germination seedlings, an analysis of the transcripts of all TPP dependent genes was conducted via qPCR (Figure.4.13). In Tkox lines the levels of *DXS* transcripts were found to be higher than WT levels with Tkox -1 and -8 showing the greatest increase at 3 days post germination. However, at 9 days all three Tkox lines had approximately half the DXS transcript levels seen in WT plants. In addition, PDC transcript levels in Tkox plants were lower than WT after growth for 3 days, but equivalent to WT at the 9 day stage. Conversely, BCKDC transcript levels were equivalent to that of WT at 3 days, but lower at 9 days.

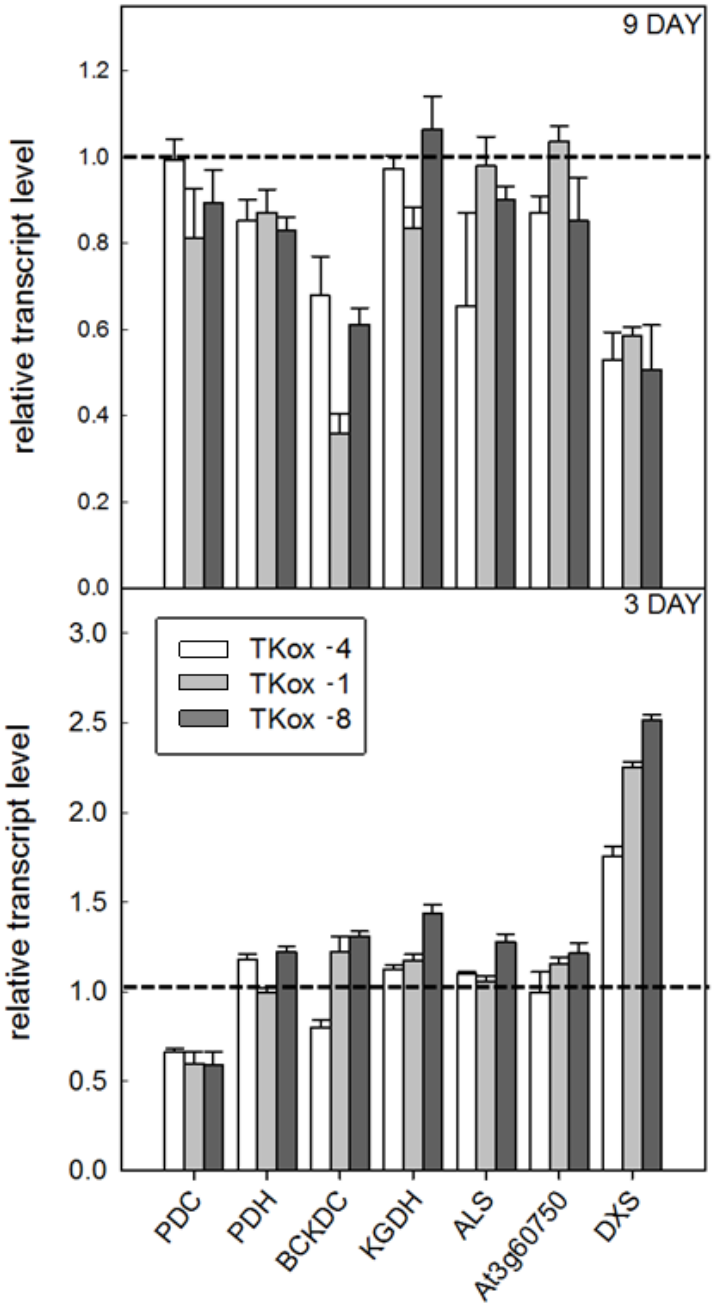

Figure 4.13- qPCR analysis of transcript levels of TPP dependent enzymes. The values represent cDNA that was comprised of equal quantities of RNA from 3 independent samples of pooled plant material from each line (WT, TK4, TK1 and TK8). PDC: pyruvate decarboxylase, PDH: pyruvate dehydrogenase, BCKDC: branched-chain a-ketoacid dehydrogenase, α-KGDH: α-ketoglutarate dehydrogenase, ALS: acetolactate synthase, NAT.TK: native plastid transketolase, DXS: 1-deoxy-D-xylulose-5-phosphate synthase. The values represent a pooled sample of whole plant material taken at 3 and 9 days post germination. The error bars indicate the S.E. from 3 technical replicates.

4.7 RNA-seq analysis of the aerial leaves of flowering plants.

To help identify pathways and/or individual genes that may be affecting thiamine metabolism in Tkox plants, RNA-seq analysis was conducted on two pooled samples (4 biological reps each from WT and Tkox -1 lines) from the aerial leaves of flowering plants. These leaves had previously been shown to have large differences in thiamine levels which coincided with the fall in seed thiamine levels of Tkox lines.

A gene ontology (GO) enrichment analysis produced from the RNA-seq results using the David bioinformatics database (Da Wei Huang and Lempicki, 2008) highlighted that genes that are active in both response to stimulus and developmental process were the two largest areas affected from variations in transcript levels of Tkox compared to WT (Figure.4.14).

However, analysis of the six genes with the largest fold differences (Table 4.1) from WT showed that the majority were involved in phenylpropanoid metabolism (Figure.4.15) whilst starch and sucrose metabolism, flavonoid biosynthesis, the shikimate pathway and plant hormone biosynthesis were also identified. Further analysis identified that 2 of these genes based on closest *Arabidopsis* homologues of tobacco genes: AT1G22410 (Class-II DAHP synthetase family protein) and AT4G34050 (S-adenosyl-L-methionine-dependent methyltransferases superfamily protein) were shown to have a correlated expression pattern in the STRING database of known and predicted protein interactions (Jensen et al., 2009) (Table.4.2).

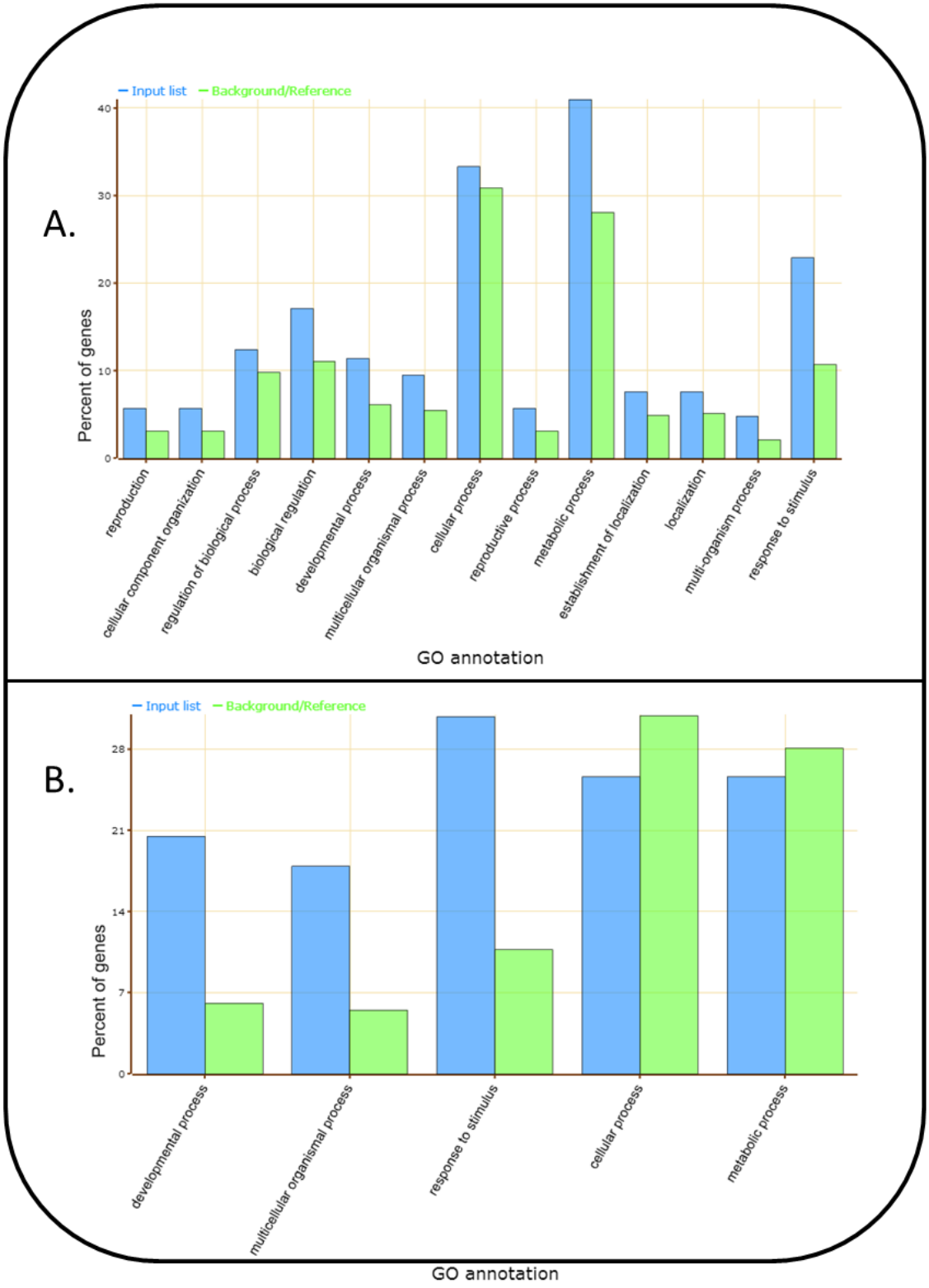

Figure 4.14- Gene ontology enrichment analysis of RNA-seq data. Seeds from WT and Tkox lines were imbibed in thiamine solution and then grown under long day conditions until the first flower opened. The youngest four leaves from each plant were sampled at mid-point through the day cycle. RNA from each plant (n=4 for WT and TKox -1 lines) were pooled and sent for RNA-seq analysis. Both upregulated (A) and down regulated (B) genes underwent an individual gene ontology analysis to identify affected biological processes. Green bars indicate the expected percentage of gene transcripts allocated between different systemic processes. The blue bars show the percentage of total gene transcripts from the RNA-seq analysis compare.

Table 4.1- The six genes identified with the largest fold differences in transcript numbers from RNA-seq analysis on the aerial leaves of Tkox flowering plants. Analysis of 2 genes that showed the largest increase in the aerial leaves of flowering TKox lines vs. WT were shown to have an association in their expression pattern with TK expression when run against the STRING database of known and predicted protein interactions (Jensen et al. Nucleic Acids Res. 2009, 37(Database issue):D412-6).

Arabidopsis gene homologue	Gene	Fold change (TKox/WT)
AT1G22410	Class-II DAHP synthetase family protein	3.89
AT5G28237	Pyridoxal-5'-phosphate-dependent enzyme family protein	0.21
AT4G29010	Enoyl-CoA hydratase/isomerase family	3.86
AT5G07990	Cytochrome P450 superfamily protein	3.36
AT4G25420	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein	2.94
AT4G34050	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	7.44

Table 4.2- The 2 most significantly affected genes in the aerial leaves of Tkox flowering plants. Analysis of 2 genes that showed the largest increase in the aerial leaves of flowering TKox lines vs. WT were shown to have an association in their expression pattern with TK expression when run against the STRING database of known and predicted protein interactions (Jensen et al. Nucleic Acids Res. 2009, 37(Database issue):D412-6).

GENE I.D	NAME	TK	WT	FOLDCHANGE (TK/WT)
AT1G22410	Class-II DAHP synthetase family protein	126	32	3.88
AT4G34050	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	6	0.8	7.43

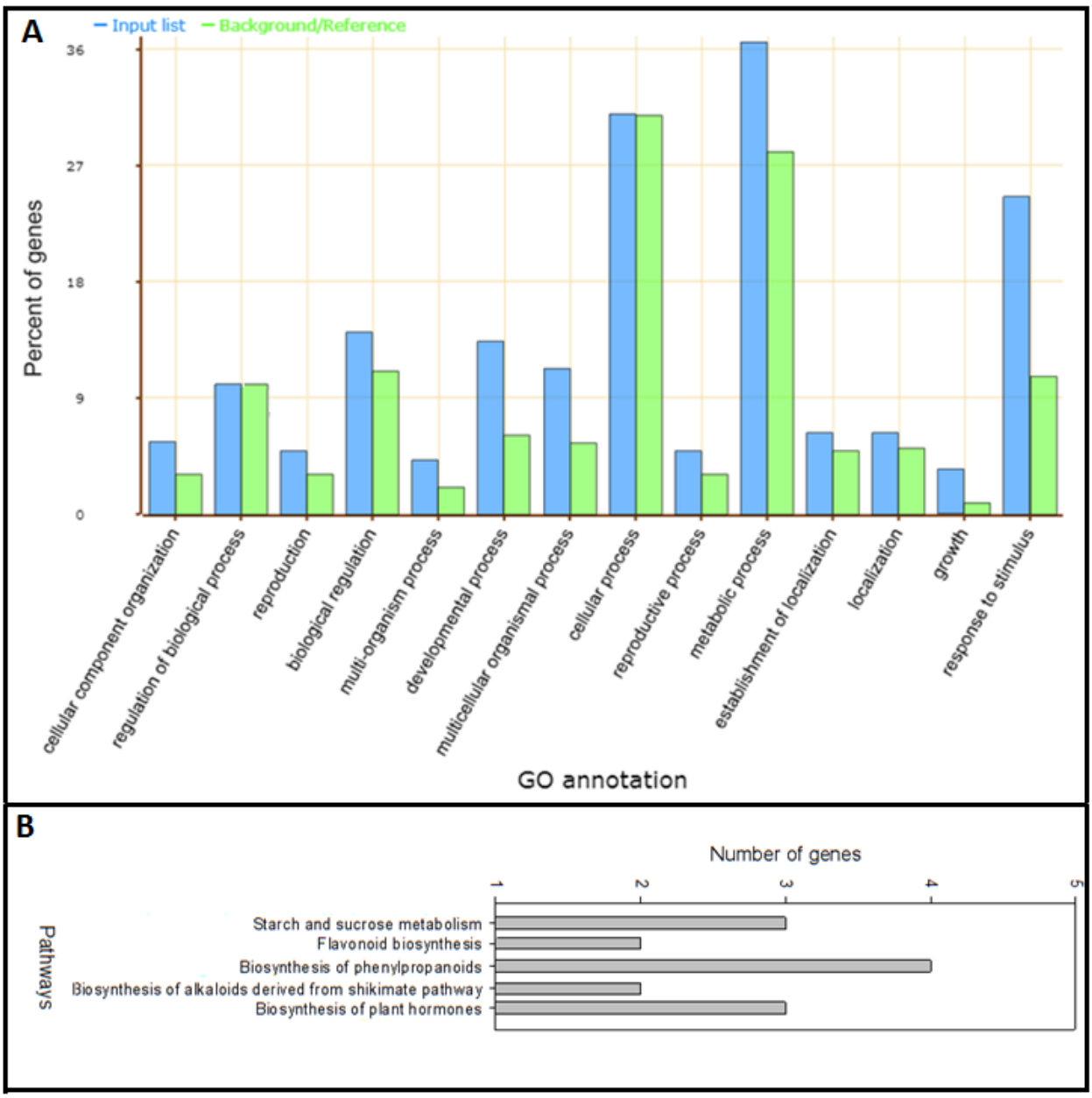

Figure 4.15- Gene ontology enrichment analysis of the 6 most altered genes in the youngest leaves of flowering plants. Seeds from WT and TKox lines were imbibed in thiamine solution to overcome the growth phenotype in T and then grown under long day conditions until the first flower opened. The youngest four leaves from each plant were sampled at mid-point through the day cycle. RNA from each plant (n=4 for WT and Tkox -1 lines) was pooled and sent for RNA-seq analysis. (A)Values signify the percentage of total genes that were found to have the largest differences from WT transcript levels. (B) Pathways affected based on the transcript levels of the genes affected.

Discussion

The results presented in this Chapter have provided evidence to support the proposal that the cause of the initial growth/chlorotic phenotype seen in TKox lines is a reduction of thiamine levels in the seeds. A thiamine storage facility is provided by thiamine binding proteins (TBPs) in the embryo which act as storage molecules during seed development (Mitsunaga et al., 1986). Upon germination, degradation of TBP's occur and thiamine is released (Watanabe 2004). The released thiamine is then converted to TPP which plays a crucial role as a co-factor for enzymes that are essential for seedling growth and establishment.

The discovery that thiamine levels in the seeds from TKox lines can be increased through either imbibing them in a thiamine solution or supplying an exogenous thiamine source to the parental plant during seed production provides evidence that the thiamine binding mechanism in the seed is not affected by the insertion position of the Tkox construct in the transformation event. Therefore, these results show that insufficient thiamine is being made available for seed production which leads to a significant reduction in their thiamine content. As a consequence of this there is a shortage of thiamine in the seed at germination which results in lower levels of thiamine availability in Tkox seedlings than in WT seedlings.

As would be expected, the reduced rate of thiamine contained in the seed was found to cause a significant change in thiamine metabolism in post-germinative seedlings. However, what was surprising was that it was thiamine and not TPP levels that were significantly reduced. This evidence shows the importance of TPP levels in seedling development as TPP levels are maintained at the detriment of thiamine suggesting that TPP levels are required to be maintained at a specific level

At present, no distinct role has been given to thiamine apart from it being an intermediate in the production of TPP. TPP is often referred to as the active form of the vitamin because TPP has the role of an enzymatic co-factor. It has been proposed that deficiency in thiamine due to a reduction in thiazole biosynthesis leads to insufficient meristem maintenance and a reduced growth phenotype (Woodward 2010). However, It was not stated in this research whether a particular form of thiamine was involved in this meristem development function but because TPP levels are found to be comparable to those levels seen in WT this may indicate that thiamine and not TPP (active form) deficiency is the cause of growth the phenotype in TKox lines.

Despite increased levels of total TK protein having been shown to have a detrimental effect on thiamine metabolism. It appears that thiamine and TPP levels are not affected uniformly throughout the life cycle but rather only at key stages of development such as in the seedlings and in the youngest leaves of flowering Tkox plants. However, because plants begin their lives as thiamine heterotrophs the shortage of thiamine in the seed is a direct consequence of thiamine availability in the parental plant during reproductive growth.

Further evidence of this lack of uniformity in changes to thiamine and TPP levels was demonstrated in 21 day old Tkox plants undergoing vegetative growth. Those Tkox plants that were receiving no thiamine supplementation (+/- and -/- treatments) showed no significant difference in the levels of either TPP or thiamine compared to WT and both thiamine and TPP levels were similar. In contrast, when the 6^{th} true leaf was sampled from plants under the same irrigation regime, TPP levels were 2 fold or higher than thiamine levels.

This suggests that thiamine metabolism in photosynthetic organisms is a highly regulated process. Mechanisms which control the biosynthesis of the pyrimidine moiety of thiamine include a light sensitive promoter region (Bocobza et al., 2013) and a riboswitch mechanism which is located in the ‘3 UTR region of nascent ThiC mRNA (Moulin et al., 2013). Changes in the availability of TPP cause alternative splicing of ThiC mRNA which controls thiamine production based on the needs of the plant (Wachter et al., 2007). Despite the finding in this chapter that TPP levels were not found to significantly differ from that seen in WT plants in the majority of cases. The levels of ThiC transcripts were found to be increased demonstrating an increased demand for TPP. Interestingly, the alternative ThiC transcript, ThiC3, indicated TPP deficiency in TKox lines by having low levels of this transcript variant. The finding that thiamine supplementation can increase ThiC3 levels suggests that TKox lines have an increased demand for thiamine but are unable to increase thiamine biosynthesis to meet demand.

The transcript levels of TPP dependent genes did show some variation in 3 and 9 day seedlings with DXS showing ~2 fold increase at 3 days post germination and a 2 fold decrease at 9 days post germination. DOXP is the first substrate in the biosynthesis of chlorophyll. One hypothesis to explain this is that carbon flow to the MEP pathway is restricted due to the increased activity of TK which negatively affects the substrates available for chlorophyll biosynthesis.

In an attempt to provide further information as to the cause of the growth and chlorotic phenotype caused by the increase in TK protein levels. A pilot RNA-seq analysis was carried out using the leaf material from flowering plants which showed large decreases in thiamine and TPP levels. Surprisingly, there were no major changes in the transcripts encoding either the TPP requiring enzymes or enzymes of the thiamine

biosynthetic pathway of TKox lines. Despite this, the RNA-seq results did highlight a possible disruption to phenylpropanoid metabolism in TKox lines which was attributed to two specific genes: DHP synthase and caffeoyl coenzyme a o-methyltransferase.

The products of the phenylpropanoid metabolic pathway are utilized in the generation of a vast number of secondary metabolites such as lignin, flavonoids and tannins. All products of phenylpropanoid metabolism originate from the amino acids phenylalanine and tyrosine which are produced via the shikimate pathway in plants. Levels of phenylalanine were found to depress production of the thiazole moiety required for thiamine biosynthesis (Kawasaki et al., 1969) whilst thiamine supplementation has been shown to alter the expression of phenylpropanoid pathway genes (Boubakri et al., 2013). In addition, TK is also linked to this process as its reactions directly involve E4P which is the first step in the shikimate pathway and phenylpropanoid metabolism. However, it is not known whether transketolase or reduced thiamine availability is affecting this mechanism in these plants.

The effect transketolase can have on this pathway and ultimately phenylpropanoid metabolism was shown in transketolase knockdown plants where decreased levels of phenylalanine were identified if transketolase activity was reduced (Henkes et al., 2001). Interestingly, in bacteria an increase in TK levels was found to cause an increase in DAHP production leading to a shortage of phosphoenolpyruvate (PEP) synthase levels. The effect of this was to create a bottleneck in the system thereby limiting the yield of aromatic metabolites (Patnaik and Liao, 1994). It is therefore a possibility that an increase in TK levels would increase phenylalanine production and possibly inhibit thiamine biosynthesis. A previous analysis of TKox lines did not indicate significant changes in phenylpropanoid metabolites (Lefebvre et al., 2005).

However, only one analysis was conducted at one time point so it is possible that important changes in phenylpropanoid metabolism may have been missed.

In conclusion, this chapter has shown that the seeds of TKox lines have lower levels of thiamine which correlates with the increase in the level of TK activity in these plants as well as significantly reduced thiamine levels in the youngest leaves of flowering plants. Interestingly the reduction in thiamine availability did not lead to a significant reduction in TPP levels in seedlings indicating that maintaining the levels of TPP in these plants leads to thiamine deficiency. However, the ThiC transcript variants indicate that these plants are actually TPP deficient indicating that the problem is more complex than just thiamine deficiency. It may possibly indicate that vegetative growth is altered depending on the availability of TPP and the activity of the enzymes that utilize it as a co-factor.

Because the plants analysed in this chapter were comprised of the entire shoot system of a number of plants taken at a chronological point rather than at a specific developmental stage it is possible that important information may have been missed, especially as younger leaves were shown to have significant increases in thiamine and TPP levels. Therefore the following chapter will study these plants based on pooled samples of individual leaves at 4 developmental stages. Because thiamine levels have been shown to increase at ~12 days post germination the stages will bridge the gap between thiamine heterotrophic and autotrophic growth which has been shown to have the largest impact on plant development

CHAPTER 5

Investigation into seedling thiamine metabolism and TK protein levels at the same developmental stages.

Introduction

The results from the previous chapter indicated that increased TK activity impacts on thiamine/TPP levels at specific stages of development of the plant. In flowering plants, a significant reduction in the level of thiamine and TPP was seen in the youngest fully expanded leaf (leaf 20) of TKox lines compared to WT plants. In addition, the seeds from these plants had a significant reduction in amount of thiamine compared to seed from the WT plants.

Thiamine but not TPP levels were found to be low across a range of developmental stages. This suggests that TPP levels are maintained albeit at the expense of thiamine. However, there were some exceptions to this such as the young leaves of the flowering plants. In this chapter to explore this further we have set up an experiment where leaf material would be tested for both thiamine and TPP at the same developmental stage. 4 stages of development were targeted as follows including emergent and expanding cotyledons and the 1st and 2nd true leaves.

- Stage 1: Cotyledons that had a reached 3mm diameter.
- Stage 2: Cotyledons where the 1st true leaf had begun to develop.
- Stage 3: Cotyledons and the 1st true leaf where the second leaf had begun to develop.
- Stage 4: Cotyledons, 1st and 2nd true leaf where the 3rd leaf had begun to develop.

These stages were chosen as they covered the time period where thiamine was provided by seed reserves (stages 1) and also where the thiamine was provided by the seedling itself (stages 2 onwards). Because the previous chapter had identified that both thiamine and TPP had been shown to vary in different leaves at the same

stage of development, this suggested that thiamine that is being transported from older to younger leaves to supply the necessary levels of thiamine/TPP required in developing tissue may be being altered.

By measuring the gene transcript levels of both the Calvin cycle and thiamine biosynthesis genes, the levels of thiamine and TPP as well as TK protein levels this chapter aims to provide further insight into the relationship between TK and thiamine metabolism.

Results

5.1 The rate of development is reduced TKox lines

The developmental growth rate of TKox lines was found to be ~ 2 days behind the equivalent WT plants in reaching the first stage of development which is when the measurement across the cotyledons reached 3mm (Figure.5.1) (Table.5.1). However, the delay in reaching this stage of development was found to be largest in the Tkox -1 and -8 lines whilst -4 lines were closest in their developmental rate to WT plants. The time taken for Tkox plants to reach the subsequent stages of development: ST.2 (Cotyledon fully expanded and 1st true leaf visible), ST.3 (2nd true leaf visible) and ST.4 (3rd true leaf visible) was found not to differ to WT plants when the initial delay to reach ST.1 was taken into account. Despite this, both the shoot diameter and shoot weight in TKox lines -1 and -8 were found to be significantly reduced at developmental stages 3 & 4 compared to WT (Figure.5.2)

Table 5.1- The difference in developmental rate of TKox lines. Seeds from WT and Tkox lines were germinated in a controlled environment chamber set at 25^{o} C, 150 µmol m^{-2} s^{-1} light levels with a 12 hour photoperiod. Leaf tissue was removed at the following developmental stages: ST.1 (Cotyledons 3 mm at widest point across both and no first true leaf evident), ST.2 (Cotyledon fully expanded and 1st true leaf visible), ST.3 (2nd true leaf visible), ST.4 (3rd true leaf visible). N=10. Mean values were rounded to the closest full day,

Days taken to reach	WT	TKox -1	TKox -4	TKox -8
Germination	4	4	4	4
Stage 1	7	8	7	9
Stage 2	14	17	14	18
Stage 3	23	28	23	29
Stage 4	35	42	35	43

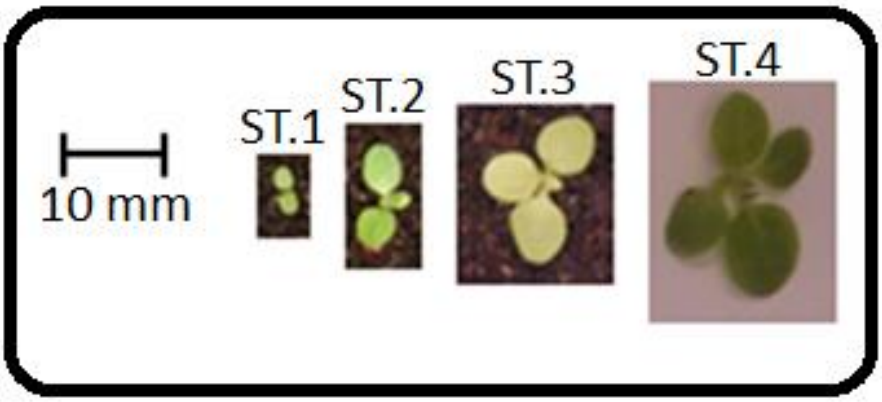

Figure 5.1- Phenotype of the 4 developmental stages. Seeds from WT and Tkox lines were germinated in a controlled environment chamber set at 25^{o} C, 150 µmol m^{-2} s^{-1} light levels with a 12 hour photoperiod. Leaf tissue was removed at the following developmental stages: ST.1 (Cotyledons 3 mm at widest point across both and no first true leaf evident), ST.2 (Cotyledon fully expanded and 1st true leaf visible), ST.3 (2nd true leaf visible), ST.4 (3rd true leaf visible).

Shoot weight P values (95% sig.)			
	TKox-1	TKox-4	TKox-8
Wt (st.3)	0.02	0.86	0.00
Wt (st.4)	0.00	0.92	0.00

Surface area P values (95% sig.)			
	TKox-1	TKox-4	TKox-8
Wt (st.2)	0.217	0.969	0.046
Wt (st.3)	0.078	0.921	0.004
Wt (st.4)	0.019	0.68	0.00

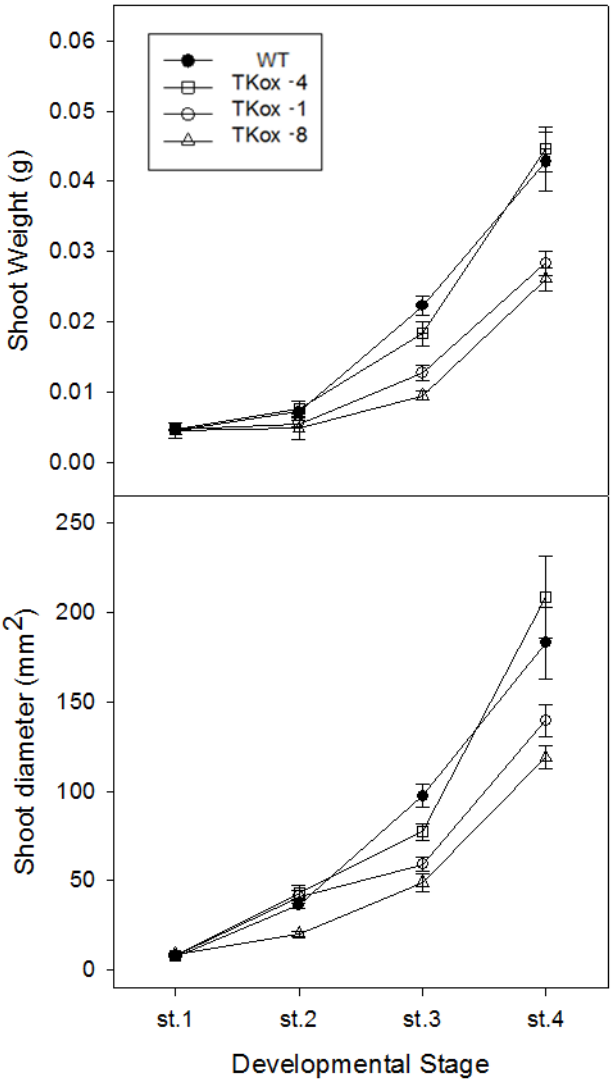

Figure 5.2-Shoot diameter and weight of WT and Tkox lines at 4 developmental stages. Seeds were germinated on compost in a controlled environment chamber set at 25^{o} C, 150 µmol m^{-2} s^{-1} light levels with a 12 hour photoperiod. Samples were taken midpoint of the photoperiod and measured for their total shoot weight and leaf surface area at 4 different stages post germination.ST.1 (Cotyledons 3 mm at widest point across both and no first true leaf evident), ST.2 (Cotyledon fully expanded and 1st true leaf visible), ST.3 (2nd true leaf visible), ST.4 (3rd true leaf visible), Statistical comparison was made using a one way Anova (95 % sig) and Tukey post hoc test, * indicates significance at P =< 0.05. Results are the mean ± SE (n = 8).

5.2 Increased TK activity influences the balance of thiamine and TPP

The balance between thiamine and TPP levels in the cotyledons, 1st and 2nd true leaves was found to show substantial variation (figure.5.3). Thiamine levels in the cotyledons of TKox lines were shown to be significantly lower than in WT plants at developmental stages 1 & 2. In contrast, TPP levels at this stage of development in TKox lines were found only to be significantly lower than WT plants in the cotyledons of stage.1 Tkox -1 plant where levels of TPP were approximately 50% that seen in both WT and Tkox -4 plants.

In the 1st true leaf, which is only present in developmental growth stages 3 and 4, TPP levels showed a small reduction and then an increase at these growth stages respectively, whilst thiamine is lower in both TKox lines compared to WT but only at developmental stage 4. A decrease in thiamine levels was also seen in TKox lines in the 2nd true leaf at developmental stage 4 where both TKOX -1ox and TK4ox lines have thiamine levels that are ~15 % of comparative WT material whilst levels of TPP are shown not to differ from that seen in WT tissue.

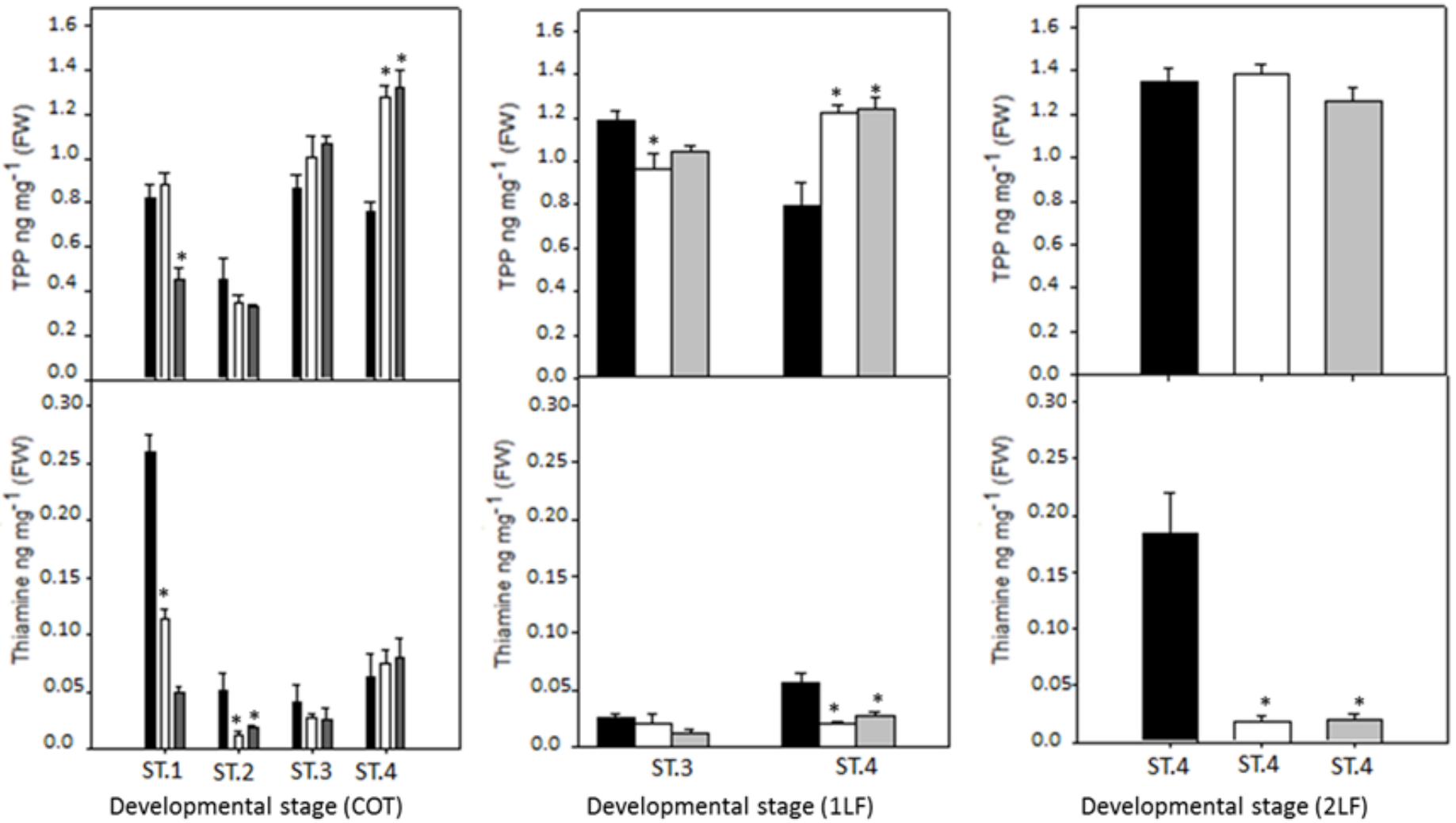

Figure 5.3- Thiamine and TPP levels in the independent leaves of WT and Tkox lines. Seeds were germinated on compost and placed in a controlled environment chamber set at 25^{o} C, 150 µmol m^{-2} s^{-1} light levels with a 12 hour photoperiod. Leaf samples were taken at the following stages: St.1 (Cotyledons 3 mm at widest point across both and no first true leaf evident), ST.2 (Cotyledon fully expanded and 1st true leaf visible), ST.3 (2nd true leaf visible), ST.4 (3rd true leaf visible), Statistical comparison was made using a one way Anova (95 % sig) and Tukey post hoc test, * indicates significance at P =< 0.05. Results are the mean ± SE (n = 4). COT= cotyledons, 1st LEAF = 1st true leaf, 2nd LEAF= 2nd true leaf

5.3 Both WT and TKox lines become thiamine autotrophs whilst the first true leaf is expanding.

Increases in the levels of both thiamine and TPP were found to occur at between the 2nd and 3rd stage of development which is indicated by the emergence of the 1st and 2nd true leaf respectively. This indicates that the plant has established the pathways necessary for production of thiamine. This result is comparable to the chronological HPLC analysis done on shoot tissue at three, six, nine and 12 days post germination in chapter 4 where 12 day shoot tissue showed an increase in both thiamine and TPP levels over those plants sampled at nine days.

5.4 TKox lines see the highest increase in the transcripts of both thiamine and Calvin cycle gene transcripts at developmental stages 2 and 4.

To identify variations in the gene transcript levels of WT and Tkox plants when at the same developmental stage a qPCR analysis was conducted on genes involved in thiamine biosynthesis (TH1, Thi1 and ThiC), the isoprenoid pathway (DXR and DXS) and the Calvin cycle (PRK and SBP) (Figure.5.4).

The results showed that the total number of ThiC splice variants (ThiC all) in TKox lines had increased transcript levels compared to WT plants at all stages and in all leaves tested with the highest levels (4 fold) seen in st.2 cotyledons and the 1st leaf at stage.4. In contrast, ThiC3 transcripts, which when expressed at low levels indicate TPP deficiency, were at their lowest in both the cotyledons and 1st leaf of stage.3 plants. TPK which phosphorylates thiamine to TPP was also shown to have similar relative increases to ThiC all transcript levels as was the dual function gene Th1 which acts as a phosphokinase and condensing enzyme to produce TMP from the pyrimidine and thiazole moieties.

The transcript levels of SBPase and PRK showed some changes to those levels seen in WT plants but these were negligible in the majority of cases. There was also little change in the transcript levels of DXS and DXR. DXS is the first enzyme involved in the MEP pathway whilst DXR commits DOXP, the product of DXS, to the MEP pathway.

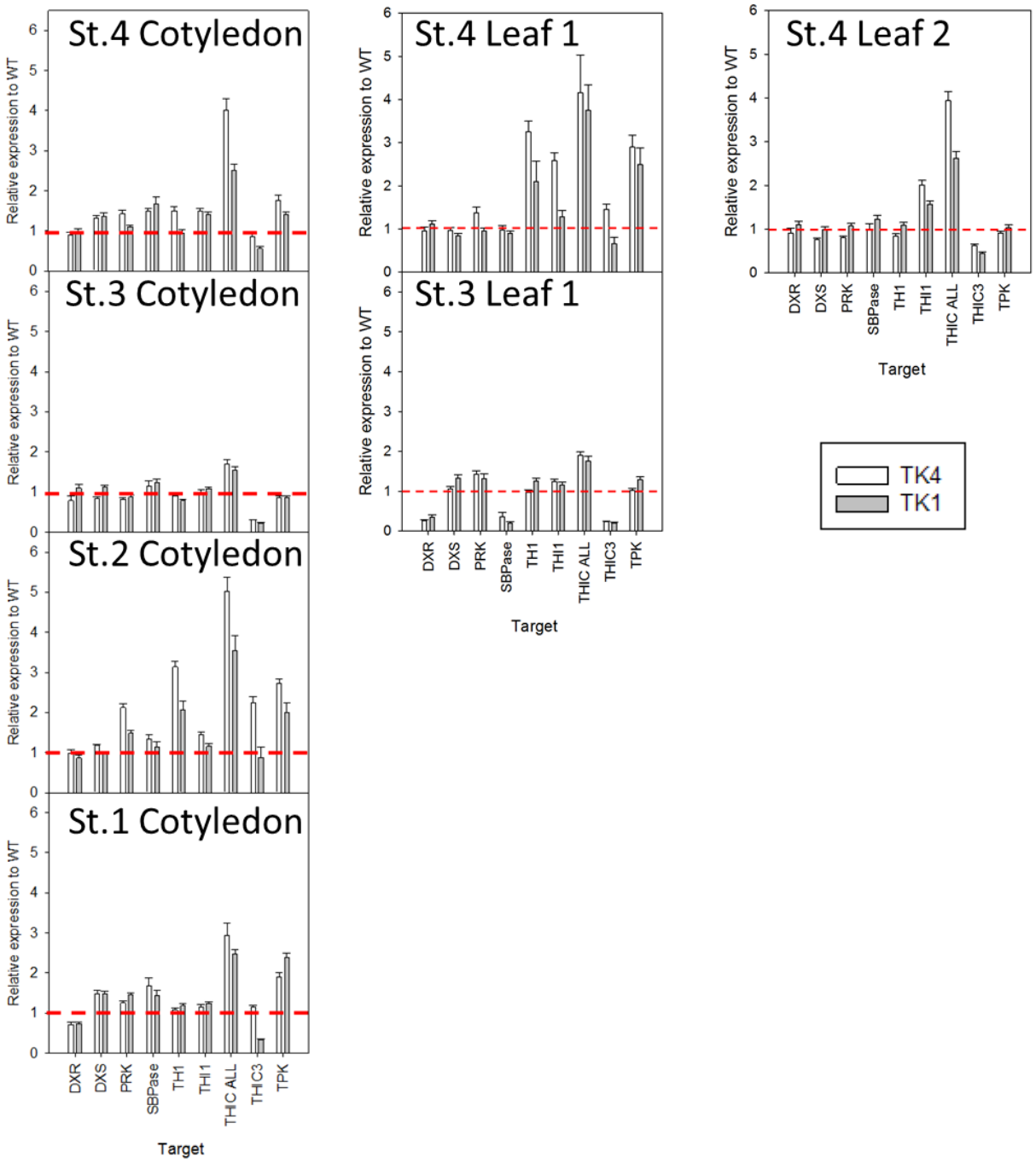

Figure 5.4- qPCR analysis of Calvin cycle and thiamine biosynthesis genes. The values represent cDNA that was comprised of equal quantities of RNA from 4 independent samples of pooled plant material from each line (WT, TKox -4 and -1). The dashed line represents the WT value. The values represent a pooled sample of leaf material removed at 4 different developmental stages: ST.1 (Cotyledons 3 mm at widest point across both and no first true leaf evident), ST.2 (Cotyledon fully expanded and 1st true leaf visible), ST.3 (2nd true leaf visible), ST.4 (3rd true leaf visible).The results are the mean from 3 technical replicates and the error bars indicate the S.E.

When the transcript levels of these genes were compared over time in the cotyledons of WT and TKox lines there was a similar pattern of over-expression between both thiamine biosynthesis genes and those involved in the Calvin cycle (Figure.5.5). Gene transcript levels were seen to rise in the cotyledons of TKox lines at developmental stage 2 (1st true leaf visible) which is followed by a fall in transcripts at stage 3 (2nd true leaf visible) and then another increase in transcripts is seen at stage 4 (3rd true leaf visible) though at this developmental period the increase in transcripts was generally less than that seen in stage 2 cotyledons. The expression levels of these same genes in the 1st true leaf of TKox lines (Figure.5.6) show that there is an increase in their expression from stage.3 to stage.4 tissues in the majority of cases. The second true leaf, which is only present at the 4^{th} stage of development, showed increases in both the thiazole producing Thi1 gene transcripts and total ThiC transcripts which produce the pyrimidine moiety of thiamine (Figure.5.7). Despite total ThiC transcripts being increased, the levels of ThiC3 were reduced in both of the TKox lines tested.

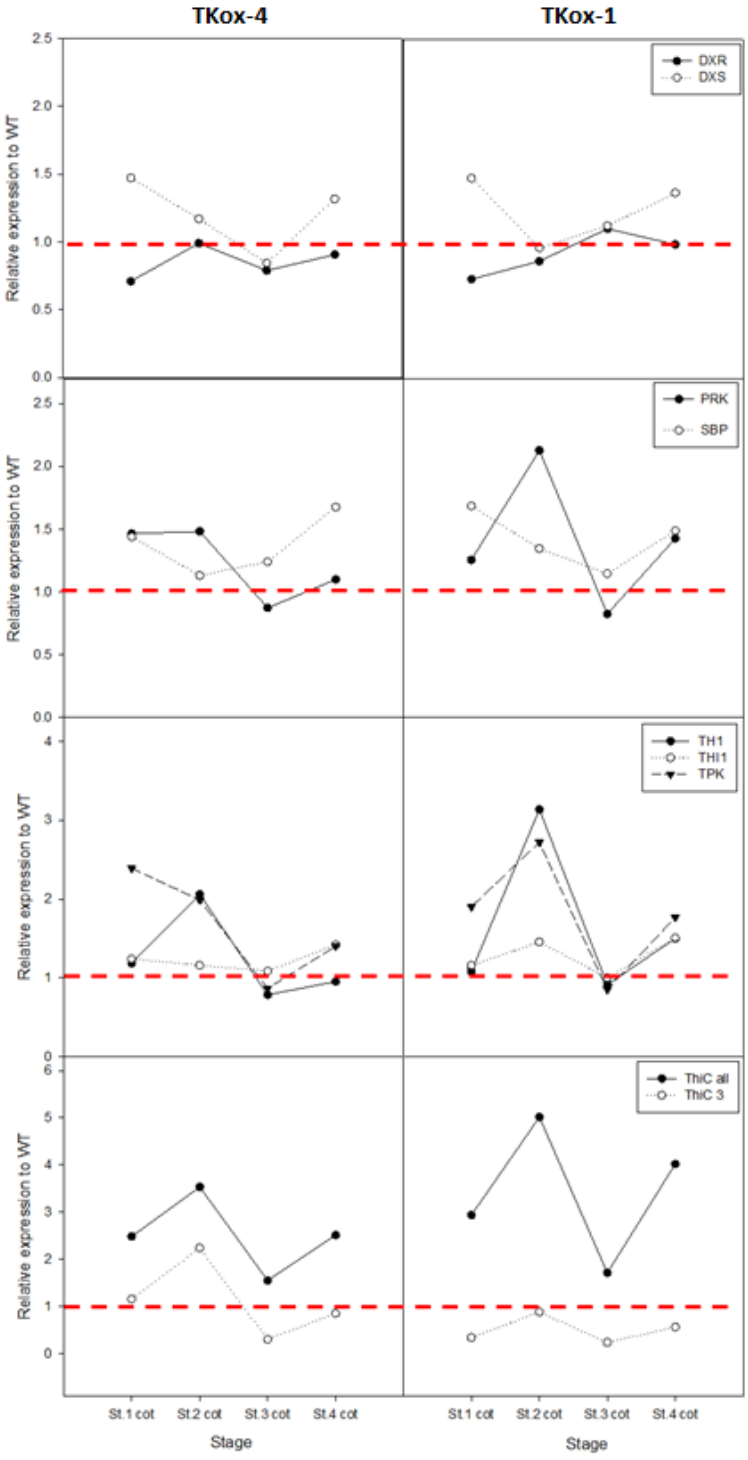

Figure 5.5- qPCR analysis of Calvin cycle and thiamine biosynthesis genes in cotyledons. The values represent cDNA that was comprised of equal quantities of RNA from 4 independent samples of pooled plant material at the following developmental stages: ST.1 (Cotyledons 3 mm at widest point across both and no first true leaf evident), ST.2 (Cotyledon fully expanded and 1st true leaf visible), ST.3 (2nd true leaf visible), ST.4 (3rd true leaf visible). The dashed line represents the WT value. The results are the mean from 4 technical replicates.

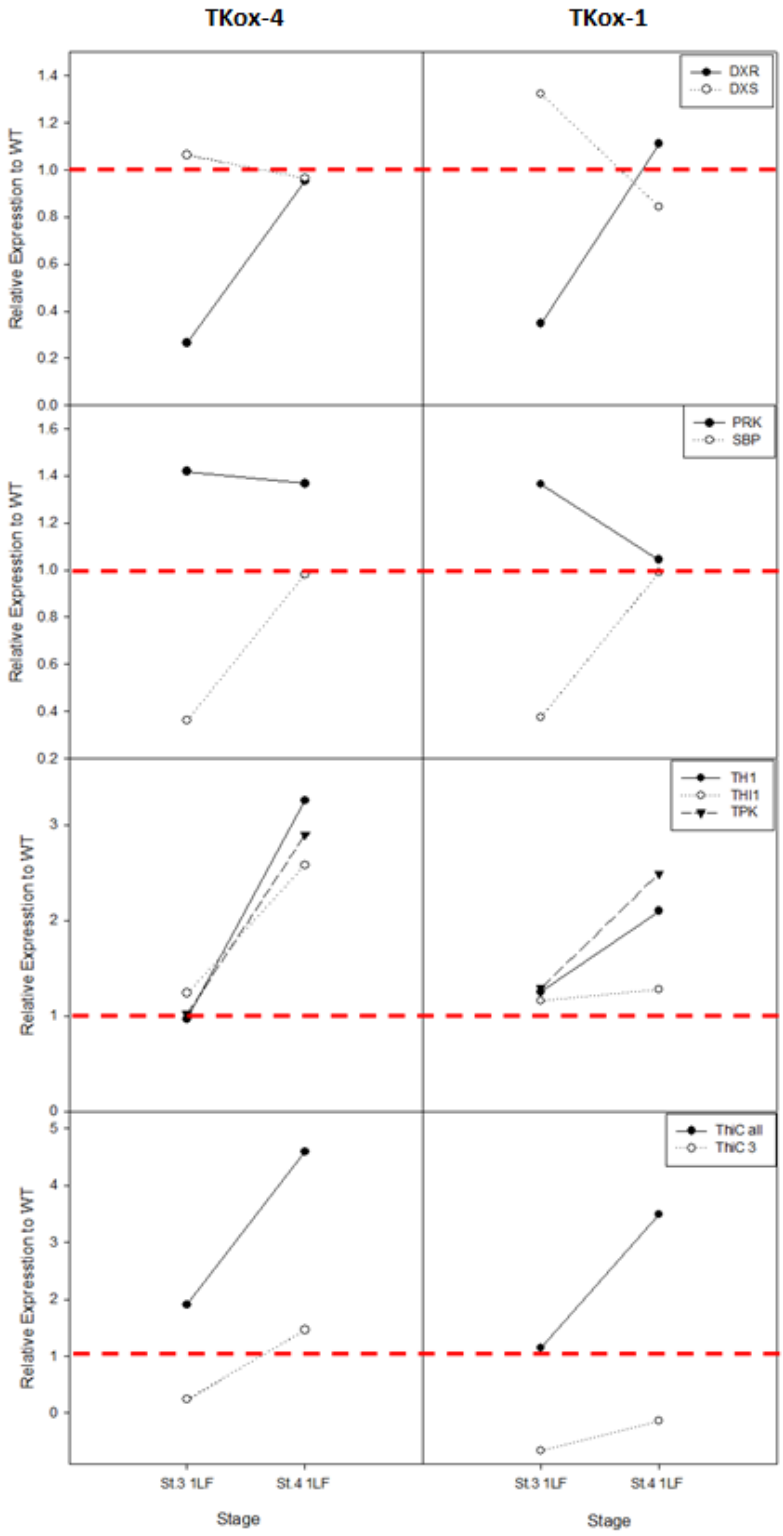

Figure 5.6- qPCR analysis of Calvin cycle and thiamine biosynthesis genes in the 1st true leaf. The values represent cDNA that was comprised of equal quantities of RNA from 4 independent samples of pooled plant material at the following developmental stages: ST.3 (2nd true leaf visible), ST.4 (3rd true leaf visible). The results are the mean from 4 technical replicates.

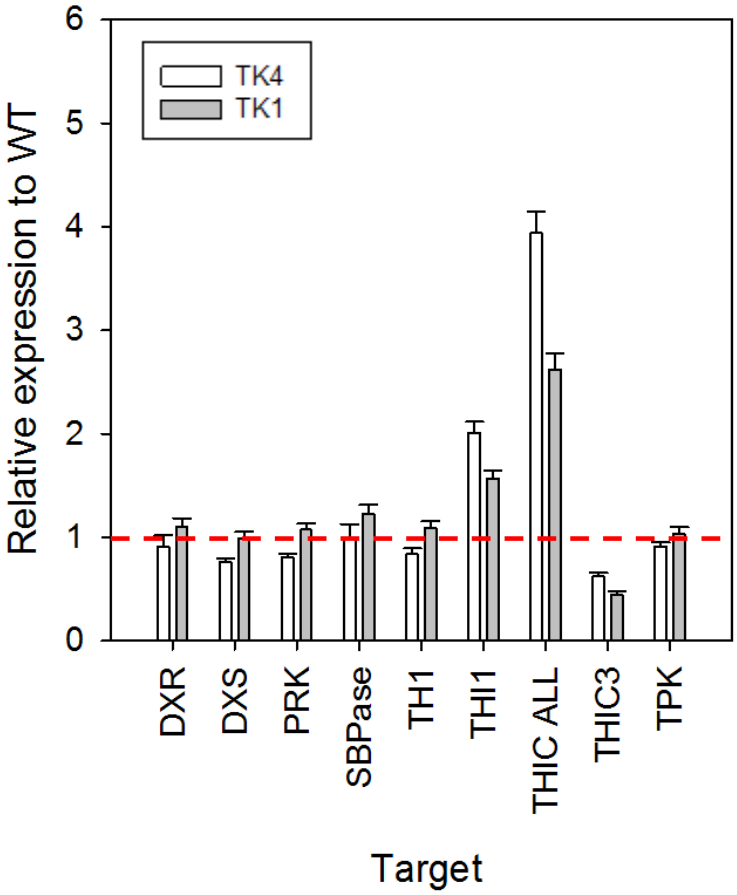

Figure 5.7 qPCR analysis of Calvin cycle and thiamine biosynthesis genes in the 2nd true leaf of St.4 plants. The values represent cDNA that was comprised of equal quantities of RNA from 4 independent samples of pooled plant material from each line (WT, TKox -4 and -1). The dashed line represents the WT value. The values represent a pooled sample of leaf material removed at 4 different developmental stages: ST.1 (Cotyledons 3 mm at widest point across both and no first true leaf evident), ST.2 (Cotyledon fully expanded and 1st true leaf visible), ST.3 (2nd true leaf visible), ST.4 (3rd true leaf visible). The results are the mean from 4 technical replicates and the error bars indicate the S.E.

5.5 The largest fold differences in TK protein levels compared to WT plants is seen in the cotyledons.

It has previously been identified that increases in thiamine biosynthesis correlate with increases seen in the transcripts of TPP dependent enzymes which include TK (Rapala-Kozik et al., 2012). However, the previous chapter showed that increases in total TK protein levels may determine how thiamine and TPP are allocated between different tissues at different stages of development.

Because of the substantial differences in both gene expression and thiamine/TPP levels in Tkox plants at the 4th stage of development it was thought that leaf material at this stage would be an ideal way to test this hypothesis.

To determine how the levels of TK protein differed between WT and TKox lines an immunoblot was conducted on the: cotyledons, 1st and 2nd true leaves in stage.4 plant. The results identified that total TK protein levels showed an increase from the cotyledon to the 1st and 2nd true leaves respectively (Figure.5.8). However, in TKox lines the level of TK protein in the cotyledon showed a threefold increase to that of comparative wild type tissue. In younger leaves, the differences in TK protein levels were still higher in Tkox lines but the fold differences compared to WT leaves were less. In comparison, PRK showed similar levels in both WT and Tkox lines regardless of leaf type or age.

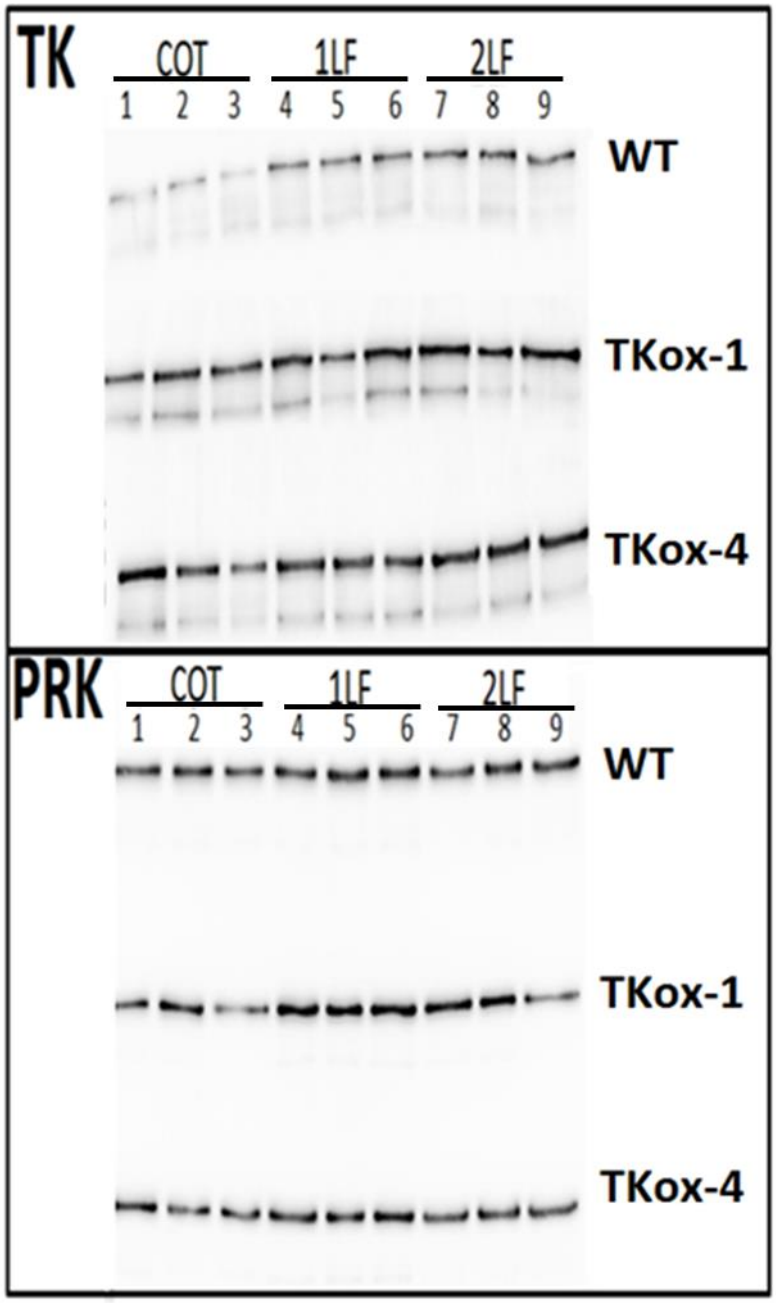

Figure 5.8- The levels of transketolase (TK) and phosphoribulokinase (PRK) protein in WT and TKox lines (-1 and -4). Protein was extracted from the cotyledons, 1st and 2nd true leaves of WT and TKox -4 -1 lines that had reached Stage.4 (the 3rd true leaf was visible) of development. PRK was used as a control. The numbers indicate biological reps of WT (1-3), TKox -4 (4-6) and TKox -1 (7-9).

Discussion

WT and TKox lines were studied for changes in the levels of TPP and thiamine at specific stages in development. During cotyledon emergence and the early expansion phase, plants are dependent on the mobilisation of thiamine supplies contained within the seed embryo until the switch to thiamine autotrophy is complete. In the previous chapter tobacco plants were shown to have an increase in the levels of both thiamine and TPP between 9 and 12 days post germination which suggests that the switch to thiamine autotrophy occurs at this stage. Furthermore, it was demonstrated that increased TK activity caused changes to thiamine metabolism at both seed development and germination which are key stages of the plant lifecycle. Therefore, by sampling leaf tissue at developmental stages that cross the thiamine heterotroph to autotroph transition the aim was to understand better the relationship between thiamine and TPP levels and increased TK activity to and to better understand the regulation of thiamine metabolism per se.

It was shown in previous chapters that there was a decrease in the growth rate of TKox lines which was attributed to a reduction in the availability of thiamine at the point of germination due to reduced thiamine levels in the seed. However, though this chapter has shown that the time taken to reach specific developmental stages is ~2 days slower in those TKox lines with highest TK activity than WT. It has also demonstrated that the shoot diameter of those same Tkox plants was found to be reduced even when both the WT and Tkox plants were at same stage of development indicating that the availability of thiamine and/or TPP was restricting growth. However without further evidence the exact mechanism can currently only be explained based on a mechanistic basis principle.

A reduction in leaf size was also found in the study of a maize thiamine auxotroph where there was a progressive reduction in leaf size as each new leave underwent development (Woodward et al., 2010). However, in that study the decrease in leaf size was attributed to truncated blade development caused by a reduction in the size of the shoot apical meristem that resulted in premature shoot abortion. The same phenotype was not present in the TKox lines tested here which suggests that another mechanism may be involved in these plants.

It was shown that the largest differences in the shoot diameter and biomass of TKox lines compared to WT plants is seen at development stages 3 & 4 which is where the first and second true leaves are developed respectively. By this stage of development, combined thiamine and TPP levels had shown significant increases over those seen in stage.2 tissues with stage.4 plant tissues combined having TPP increases of ~130% compared to WT and yet despite this the ThiC riboswitch splice variants indicated a TPP deficient phenotype in all leaf tissues indicating that there is a limitation to the level of thiamine compounds than can be made by the Tkox plants despite the increased demand indicated by the TPP riboswitch mechanism.

Alternatively a homeostatic feedback mechanism may be in place that sacrifices the growth rate to permit TPP levels to be maintained within a specific range. In addition, to the slowing of growth used to achieve this level of TPP, thiamine levels may also be sacrificed to produce sufficient TPP levels. This is supported by a study in Arabidopsis in which the mutation of the ThiC riboswitch was found to have a negative effect on homeostasis, causing chlorosis of the leaves, reduced growth, and delayed flowering. These characteristics were attributed to an increase in the activity levels of TPP dependent enzymes though TPP levels were only shown as having relatively small increases of ~20 % at most (Bocobza et al., 2013) which is comparable to the

TPP increases seen in TKox lines in the majority of cases. TK has been identified as a key regulator of central metabolism in plants (Henkes et al., 2001) (Rocha et al., 2014) which may explain why TKox lines have comparable phenotypes. Together these data may indicate that there is a maximum limit in the amount of TPP that is able to be produced in plants and to make Tkox plants viable this limitation would need to be bypassed, possibly through the development of a multi gene construct to increase TPP levels beyond what is seen here.

An alternative theory as to the TPP deficiency in TKox lines indicated by the ThiC riboswitch is that TPP molecules are being sequestered by the increased level of TK protein present which are limiting the availability of the TPP co-factor for general metabolism. The affinity of TK protein for its TPP co-factor has been found to be highly variable across species. Mammalian TK affinity for TPP was found to be strong enough to allow the enzyme to be purified and undergo subsequent activity analysis without the need for additional co-factor to be added to the reaction solution (Esakova et al., 2004). Whilst in yeast TK, the TPP co-factor was able to be removed with a moderate decrease in pH level. In addition, both the donor substrate and metal ion availability (both Xu5P and Mg^{2+}) were also shown to affect the affinity between TK and TPP (donor substrate regulation of TK (Esakova et al., 2004). If the increase in total TK protein levels was binding up excess TPP the effect of this would be to limit the amount of TPP available to be utilized as a co-factor by TPP dependent enzymes as well as regulate biosynthesis of the pyrimidine moiety by binding to the 3' UTR of ThiC mRNA leading to a decrease in ThiC 3 splice variants.

However, Calvin cycle enzymes have been shown to be maintained at levels of 10 fold or higher than those enzymes involved in primary metabolism and more so compared to pathways like the MEP pathway (Sulpice et al., 2010; Baerenfaller et al., 2012;

Mettler et al., 2014). The levels of TPP measured in TKox leaf tissue was shown to vary between ~1 and ~6 nmol g^{-1} FW whilst TK binding sites have been estimated at 3.4 nmol g^{-1} FW in spinach leaves (Harris and Königer, 1997) and 2.5 - 4.4 nmol g^{-1} FW in Arabidopsis rosettes (Piques et al., 2009). Because of the variations seen in TPP levels at different tissues at different stages of development this information may indicate that TPP deficiency is caused by the increase in TPP binding sites. Increased concentrations of TPP have been shown to increase the activity of the TPP dependent enzymes in both mammals (Fournier and Butterworth, 1990) and plants (Bocobza et al., 2013). However, the transcript levels of these enzymes show little change even when TPP levels are increased (Rapala-Kozik et al., 2012) suggesting that the control of TPP levels may determine their activity rather than substantial increase in the amount of enzyme.

It was suggested in the previous chapter that the transport and accumulation of thiamine variants between tissues may be key to understanding the mechanism behind the phenotype in TKox lines. The rationale for this is that despite TPP levels in TKox lines being equal to or higher than comparative WT plants in the majority of cases. The proportion of the TPP increase in the different leaves of stage.4 plants in Tkox plants compared to WT showed that in older leaves the percentage of TPP increase is greatest. For example, levels of TPP were shown to be ~ 160% (cotyledons), ~145% (1st leaf), ~104% (2nd leaf) whilst levels of thiamine showed a decrease from the oldest to youngest leaves where thiamine levels were as low as ~10% of comparable WT plants in the 2nd true leaf at developmental stage.4. At developmental stage.4 the transcripts of the thiamine biosynthesis genes: TH1, Thi1, ThiC were highest in the 1st leaf whilst the transcripts for TPK were at their highest expression level across all tissues at all stages of development. Because TPK protein

acts to phosphorylate thiamine to TPP this may indicate that TPP is produced in the first leaf and then transported elsewhere.

It has been suggested that TPP dependent enzymes such as TK may be part of a stress sensing mechanism where increases in TPP dependent enzymes are thought to lead to similar increases in the thiamine biosynthesis genes and subsequent increases in thiamine and TPP which are then able to function in protection from biotic and abiotic factors (Rapala-Kozik et al., 2012).

Some support for this hypothesis is shown here where it was found that in WT plants, TK protein levels were highest in the 2nd true leaf with clearly visible decreases in protein seen in the 1st true leaf and the cotyledons. Whilst In TKox lines the levels of TK protein were shown to be uniform across all leaves. However, because of the changes to TK levels seen in WT plants this led to differences in TK levels that were approximately two fold the level seen WT plants in the 2nd true leaf whilst in the cotyledons this difference was ~ 4x. Therefore, leaves with higher TK levels led to a higher proportional accumulation of TPP in the cotyledons and a subsequent reduction in the availability of thiamine in the leaves at the top of the plant.

In conclusion, this chapter shows that the increases in both thiamine and TPP levels seen upon the development of the first true leaf may indicate that *Nicotiana tabacum* plants become thiamine autotrophs at this stage. This is supported by the increase in the levels of thiamine biosynthesis gene transcripts: Thi1, TH1 TPK and ThiC in the cotyledons and the increased level of thiamine and TPP. Interestingly, it was found that once thiamine and TPP levels were shown to increase, the rate of growth based on the time to reach the specific developmental stages are the same as WT plants, though the leaf area is in Tkox plants is reduced. This may be due to an as yet

unidentified mechanism that regulates TPP levels by reducing thiamine levels and growth.

The levels of TPP in Tkox leaf tissue at the same developmental stage were found to be equal to or higher than WT in the majority of cases whilst levels of thiamine were found to be reduced. The same characteristic was also shown in leaf tissue sampled on a chronological basis in chapter.4. However, an immunoblot analysis of the cotyledons, 1st and 2nd true leaves showed that total TK protein levels in WT plants shows a gradual decrease from younger to older leaves. In contrast, the level of TK protein in TKox lines was consistent across all leaf tissue types regardless of their age. This meant that total TK protein levels in Tkox plants were found to be largest in the cotyledons where TPP levels were found to be highest. This finding may indicate that increased TK levels may specify areas of high TPP demand. Increases in TK gene transcripts in stressed Arabidopsis plants occurred within two hours after being placed in stress conditions (Rapala-Kozik et al., 2012). One hypothesis may be that increased TK activity may lead to the transport of TPP to the cotyledon which was shown to have the highest proportional increase in total TK protein.

CHAPTER 6- General Discussion

6.1 Overall aim and main results.

A previous study (Khozaei, 2010) showed that an increase in the level of non-native plastid TK in *Nicotiana tabacum* causes a significant reduction in plant growth and a chlorotic phenotype. Both of these phenotypes were able to be rescued through the availability of an external thiamine source which demonstrated that thiamine auxotrophy in plants can be caused by ectopic overexpression of a solitary TPP dependent enzyme. This characteristic appears to be a unique feature of TK activity levels as overexpression of SBPase, FBP aldolase, or a cyanobacterial bi-functional FBPase/SBPase did not have this negative effect (Miyagawa et al., 2001; Lefebvre et al., 2005; Uematsu et al., 2012). Therefore, the aim of this project was to further advance the understanding of the mechanism behind the increase in TK protein levels and its effect on thiamine metabolism. To achieve this, three lines from the T3 generation of TK sense plants with total TK activity levels ~2 and 2.5 that of WT plants were analysed for their biochemical and physiological properties.

It was found that the severity of the phenotype was comparable to the level of increase in TK activity levels. Tkox plants with the highest levels of TK activity (Tkox -1 and -8) had more severe phenotypes than the Tkox -4 plants which had TK activity levels that were most similar to TK activity levels seen in WT plants.

These results along with that from the *TK* antisense study (Henkes et al., 2001) have revealed that plants can tolerate only small changes in the level of the endogenous TK protein otherwise this can affect the pathways that receive compounds from the Calvin cycle. In the case of the antisense plants, a 20-40% decrease in TK activity leads to reduced levels of shikimate and phenylpropanoid pathway products (Henkes et al., 2001) whilst in TK overexpressing lines an approximate 2 to 2.5-fold increase in TK

activity leads to insufficient thiamine levels being produced to meet demand. This suggests that an as yet unknown regulatory mechanism exists that maintains TK levels within a narrow range to maintain plant homeostasis.

The chlorotic and growth phenotypes were previously thought to be two parts of a single phenotype (Khozaei, 2010). However, this research has shown that though both phenotypes are due to the Tkox plants having insufficient thiamine levels to meet demand, the phenotypes are actually independent of each other.

The reduced growth phenotype was found to be attributed to a reduction in the levels of thiamine contained within the seed at the point of germination. This leads to a reduction in the availability of thiamine compounds during the initial stages of seedling development where metabolism is dependent on sufficient thiamine levels to permit the mobilization of seed energy reserves until the switch to thiamine autotrophy is complete(Golda et al., 2004). In contrast, the chlorotic phenotype was able to be recovered if an exogenous thiamine source is supplied. If thiamine supplementation was withdrawn then the chlorotic phenotype would re-appear indicating that chlorophyll biosynthesis is interrupted unless a continuous exogenous supply is made available to Tkox plants throughout their lifecycle

6.2 The non-melovanate pathway.

One explanation for the chlorotic phenotype is that the production of DOXP from the condensation of G3P and pyruvate by the enzyme 1-deoxy-d-xylulose-5-phosphate synthase (DXS) is inhibited. DOXP is the first intermediate in the production of isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) which are the fundamental building blocks of the phytol moiety of chlorophyll.

These data have provided evidence of the role of DXS in this phenotype through the discovery that supplementation of TKox lines with DOXP is able to recover the chlorotic phenotype in Tkox seedlings. This suggests that the rate of flux through the DXS is affecting the production of chlorophyll. Similar chlorotic phenotypes as shown here have been identified in DXS mutants where the chlorotic phenotype was also able to be complemented with an exogenous source of DOXP (Araki et al., 2000).

DOXP is known to be a substrate that is used in the biosynthesis of the thiazole moiety of thiamine in bacteria, whilst in eukaryotes, including plants there is evidence that the thiazole moiety is produced from the condensation of glycine and NAD+ (Chatterjee et al., 2007). However, it has also been shown that extracted chloroplast stromal proteins can produce the thiazole moiety when they are supplied with pyruvate, glyceraldehyde 3-phosphate, tyrosine, cysteine, and MgATP (Julliard and Douce, 1991b). Because of the uncertainty of the route for biosynthesis of the thiazole pathway in plants it would be interesting to measure the levels of the thiazole moiety to determine if its production is affected.

6.3 Thiamine and TPP levels.

The levels of thiamine and TPP determined at different stages of development showed that increased TK activity leads to a thiamine but not TPP deficiency in the majority of the tissues analysed in this research. Thiamine levels in the leaves of TKox lines had thiamine decreases of up to 90% that of WT plants which suggest that TPP levels are being maintained at the expense of thiamine, possibly to maintain flux through pathways that utilize TPP dependent enzymes.

Despite TPP levels in TKox lines being equal to or higher than in WT plants, the regulatory ThiC riboswitch mechanism indicated a TPP deficient phenotype. Though the reason for this remains unknown it may be due to the increased level of TK protein sequestering TPP molecules and thereby preventing their use by the other TPP dependent enzymes. However, because transcript levels of TPP dependent enzymes did not show significant increases in the majority of cases this seems unlikely.

Another possibility is that the transport of TPP between cellular compartments is affected thereby limiting TPP access to the regulatory ThiC riboswitch mechanism. TPP is the co-factor for a number of enzymes that reside in both the chloroplast and the mitochondria. In plants only mitochondrial TPP transporters have been identified (Frelin et al., 2012). Although, transporters also exist in the chloroplast as thiamine is synthesized in chloroplasts (Belanger et al., 1995) and needs to be transported to other cellular compartments. One of these is the nucleus where TPP binds to the 3' UTR of ThiC nascent mRNA which regulates the production of the ThiC protein and ultimately the production of thiamine. If TPP was unable to access the nucleus and bind to the riboswitch mechanism of ThiC then it would be expected to produce a similar result as shown here.

One question highlighted by this work is that if thiamine/TPP levels are insufficient to meet demand then why is not the rate of thiamine biosynthesis increased to meet demand? Based on the results from this study as well as that in the literature, it would appear that TPP levels are limited to a maximum level in plants. In plants that have been grown under stress conditions (Rapala-Kozik et al., 2012) and also in plants with a manipulated ThiC riboswitch mechanism (Bocobza et al., 2013) an approximate 20% TPP increase was seen in both cases. Interestingly TKox lines also showed similar increase to the levels of TPP though sampling of independent leaves in

seedlings did indicate increases in TPP of up to 40% suggesting that sampling of whole plants may mask variations in the levels of thiamine and TPP.

An increase in total thiamine levels in plants is a desirable trait as increased thiamine has been shown to provide protection from plant disease (Ahn et al., 2005). Whilst crops with increased thiamine content would prevent the occurrence of human diseases related to thiamine deficiency(Pourcel et al., 2013). It was suggested previously that the finding that DOXP was able to partially complement the phenotype may indicate that insufficient biosynthesis of the thiazole moiety may be the reason that Tkox plants are unable to produce sufficient levels of thiamine in TKox lines.

6.4 The accumulation of TPP in areas of high TK activity.

It has been known since the 1960's that thiamine is transported from the shoot to root system (Addicott, 1957) whilst more recent research has indicated that the pyrimidine and thiazole moieties that are combined to create thiamine may be transported independently to the developing seed embryo to provide sufficient thiamine levels for the next generation (Guan et al., 2014).This study has provided evidence to suggest that the level of TK activity may impact the accumulation and possibly the availability of TPP for transport between tissues.

In plants that had begun to flower, TKox lines had reduced thiamine and TPP levels in the 20th fully open leaf compared to the same leaf in WT plants at the same developmental stage. In addition, the seeds produced from these Tkox plants had a ~50% reduction in their thiamine content. This caused a slower developmental rate with reduced vegetative growth and delayed flowering.

Further evidence for the effect increased TK activity may have on thiamine availability and accumulation was shown in the analysis of the cotyledons as well as the 1st and 2nd true leaves when plants were at the same developmental stage. TPP levels in the cotyledons were ~ 40% higher in TKox lines than WT plants. Whilst thiamine levels in the 2nd true leaf was ~10% of that seen in 2nd true leaf from WT plants. Interestingly, the largest difference in TK protein levels in Tkox plants compared to WT was in the cotyledons. This evidence suggests that tissues containing high levels of TK protein may indicate areas of high TPP demand leading to thiamine shortages in other parts of the plant which affects growth and development.

6.5 Final conclusions and future work.

The findings in this study have shown that plants can tolerate only small changes in the level of the endogenous TK protein without detrimental effects on metabolism (Henkes et al., 2001). Further evidence for this has been shown in a recent study on *Cucumis sativus* Tkox plants where much smaller increases in TK activity have been shown to have a beneficial effect on carbon assimilation and growth (Bi et al., 2013). Indicating that the changes to thiamine metabolism caused by increased TK activity are specific to the Tkox plants used here. Furthermore, it has shown that excessive changes on the activity level of Calvin cycle enzymes can have detrimental effects on pathways requiring Calvin cycle intermediates.

To advance this study it is suggested that the levels of the intermediates involved in the biosynthesis of both the pyrimidine and thiazole moieties are analysed as it was suggested here that thiazole biosynthesis may be limited in Tkox plants. In addition, an inducible TK construct inserted into plants along with the use of C^{14} labelled

thiamine in supplementation experiments at the key stages of germination and seed development could be used to identify changes in thiamine metabolism and how increases in the level of TK protein affect this process. This research would provide a greater understanding of both the regulation of thiamine metabolism and the influence that TK levels have on it.

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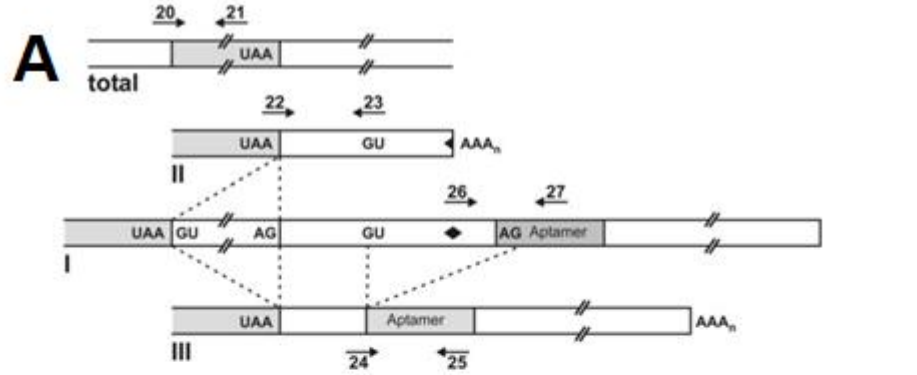
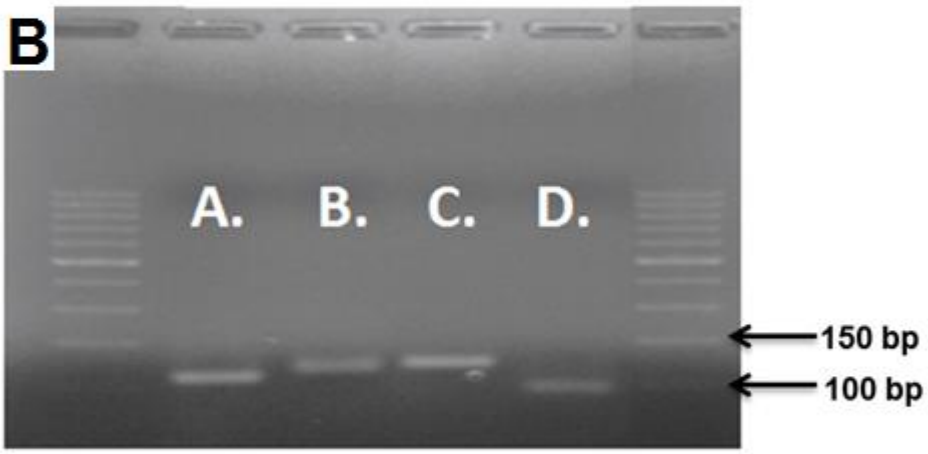
APPENDICES

Appendix 1. List of primers used. 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).

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		





lane		forward	reverse	length BP
A	ThiC	gttatggaagtgccgagga	attcaccaccaacctcacc	115
B	ThiC_3	aagtgttaggggtgcctgtg	aaaatgcacactccctacgc	130
C	ThiC_2	ctctatgaaaagctaaaggacttc	aacctagatacttacctaacacttg	135
D	ThiC_1	gacacgagaaagcaccagcg	agcctgttcagtcagcgtat	83

Appendix.2.- Primers for ThiC analysis. Primers for the independent ThiC splice variants were produced using sequences produced by Wachter et al., 2007 (A). Products of qPCR analysis were run on a 3% agarose gel (B)