UNIVERSITY OF CALIFORNIA, SAN DIEGO

An investigation into the role of TZP as a potential intermediate between light signaling and gene regulation

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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The Thesis of Olga Batalov is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

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LIST OF ABBREVIATIONS

AD – GAL4 activation domain (pDEST22) used in yeast two hybrid

AGO2 – Argonaute 2

ATHB2 – *Arabidopsis thaliana* homeobox protein 2, also known as HAT4

ATHB23 – *Arabidopsis thaliana* homeobox protein 23

BiFC – bimolecular fluorescence complementation, finds direct interactions *in vivo*

CCA1 – circadian clock associated 1

Col – Colombia ecotype, wild-type

CRY1 – cryptochrome 1, blue light photoreceptor

CRY2 – cryptochrome 2, blue light photoreceptor

C1C2P1 – cry1-cry2-phot1 triple mutant line

DBD – GAL4 DNA-binding domain (pDEST32) used in yeast two hybrid

DCL1 – Dicer-like 1, involved in miRNA processing

DRB1 – double-stranded RNA binding protein 1, also called HYL1

E22 or E32 – empty pDEST22 or pDEST32 vector

EAR – ERF-associated amphiphilic repression

ELF3 – early-flowering 3

ERF – ethylene-responsive element binding factor

GI – GIGANTEA, promotes flowering under long days, circadian clock regulation

GFP – green fluorescent protein (tag)

HDA19 – histone deacetylase 19

HDA22 – HDA19-pDEST22 (used for yeast two hybrid)

HFR1 – long hypocotyl in far-red 1

HUD – hormone up at dawn, promoter element

HYL1 – hyponastic leaves 1, also called DRB1

HYL22 – HYL1-pDEST22 (used for yeast two hybrid)

IAA1 – indole-3-acetic acid inducible 1

LHY – late elongated hypocotyl

LKP2 – LOV Kelch protein 2

LKP22 – LKP2-pDEST22 (used for yeast two hybrid)

LOV – light-oxygen-voltage

mCherry – red fluorescent tag developed in Dr. Tsien's lab

NERD – needed for RDR2-independent DNA methylation

Nt – N-terminus of TZP, truncation line

Nt32 – N-terminus of TZP in pDEST32 (used for yeast two hybrid)

PBY – dominant gain-of-function PHYB mutant (Y276H)

pDEST22 or pDEST32 – destination vectors used in Gateway cloning

PHOT1 – phototropin 1, blue light receptor in plasma membrane

PHYA – phytochrome A, far-red light signaling

PHYB – phytochrome B, red light signaling

phyA&phyB – phyA-phyB double mutant line

PIF – phytochrome interacting factor

pif4&pif5 – pif4-pif5 double mutant line

PLUS – truncation line with just PLUS3 domain of TZP

PLUS32 – PLUS3 domain of TZP in pDEST32 (used for yeast two hybrid)

PRR5 – pseudo-response regulator 5

RDR2 – RNA-dependent RNA polymerase 2

Rm32 – TZP EAR mutant (LRLRL→LALAL) in pDEST32

RTF1 – Paf1/RNA polymerase II complex component

SAP18 – SIN3-associated protein of 18 kDa

SAP22 – SAP18-pDEST22 (used for yeast two hybrid)

SE – SERRATE, involved in miRNA processing

SE22 – SE-pDEST22 (used for yeast two hybrid)

SIN22 – SIN3-pDEST22 (used for yeast two hybrid)

TPL – TOPLESS, corepressor associated with EAR motifs

TPL22 – TPL-pDEST22 (used for yeast two hybrid)

TOC1 – timing of CAB expression 1, circadian clock component

TZP – tandem zinc knuckle PLUS3 domain

TZP32 – TZP-pDEST32 (used for yeast two hybrid)

TZPearMut32 – TZP EAR mutant (LRLRL→ARARA) in pDEST32

TZP-OX – lines overexpressing TZP under 35S promoter in Col background

UGPase – UDP-glucose pyrophosphorylase, used as loading control for western blots

WT – wild-type (Col ecotype)

ZFPL – same as ZF-PLUS

ZF-PLUS – truncation line with tandem zinc knuckle and PLUS3 domains

zlf – Zeitlupe family triple mutant line

ZTL – Zeitlupe protein, also applies to family of related proteins (ZTL, FKF2, LKP2)

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Some of Eirini's work, as of yet unpublished, is included in this thesis, including the work previously done on TZP discussed in the introduction (figures 2 and 3) as well as work done on the localization and expression of the EAR mutant of TZP mentioned in the discussion.

I would also like to thank Carl Procko for helping me troubleshoot the generation of the EAR mutant of TZP (refer to materials and methods), for providing feedback on my presentation for my defense and for letting me use his laptop during my defense. I would also like to thank Ullas Pedmale for providing feedback on presentation as well as for reserving the LED light chambers for my experiments. I would also like to thank Jack Bolado and Tsegaye Dabi for helping me find or order the supplies I needed. I would like

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ABSTRACT OF THE THESIS

An investigation into the role of TZP as a potential intermediate between light signaling and gene regulation

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Master of Science in Biology

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TZP is a single copy gene in Arabidopsis with orthologs in many other plant species. It encodes a unique protein with both a PLUS3 domain and two tandem zinc knuckle domains, which have been implicated in nucleic acid and protein binding. When

overexpressed, it has a morning-specific hypocotyl elongation phenotype in blue light and acts downstream of photoreceptors and the circadian clock, but not much is known about its function or protein stability. Some work has been done to determine what proteins interact with TZP, how its localization changes under different light conditions and what functions its domains may serve.

The goals of my project were to explore how TZP protein levels are affected by different light conditions and in different mutant backgrounds as well as to investigate whether yeast two hybrid analysis could reveal any new interactions of TZP with proteins that can affect gene expression, in the hopes of shedding some light on its function. TZP was shown to accumulate most significantly after exposure to blue light, due in part to increased stability of a protein that appears to be targeted for proteasome degradation in the dark. Components of the blue light signaling pathway were shown to be important for stability in blue light and/or degradation in the dark. Yeast two hybrid analysis revealed interactions with some known EAR motif associated corepressors (SAP18 and TPL) as well as with proteins involved in miRNA processing (SERRATE and HYL1) and with the blue light receptor LKP2.

Introduction

Plant responses to light

Light perception is very important to plants. Since plants cannot move, they must be able to perceive and respond to changes in their environment in order to survive. The intensity and quality of light can tell plants much about their environment. When seedlings first germinate, they are usually in complete darkness and, in the case of dicots like the model organism *Arabidopsis thaliana*, elongate their hypocotyl (etiolation) as they search for light, using gravity to direct their growth. These dark-grown, skotomorphogenic seedlings also have closed cotyledons, an apical hook, and non-photosynthetic plastids called etioplasts (Ma L 2001). Once they detect light, de-etiolation and photomorphogenesis can occur, whereby hypocotyl elongation stops, cotyledons open, chloroplasts develop and chlorophyll is synthesized. Plants can also detect the direction of the light source and move towards it through a process called phototropism (Pedmale UV 2010; Liscum E 2014). Plants can also perceive the proximity of neighboring plants by the ratio of red to far-red light. The length of the day can be determined by detecting changes in the intensity of different wavelengths of light and controls flowering time as well as the cycling of proteins involved in the circadian clock, which controls time-of-day dependent growth cycles.

Light signaling through photoreceptors

Light signaling occurs through perception of red, far-red, blue and UV light frequencies (Lin 2002). Perception of red and far-red light occurs via the phytochromes, predominantly PHYA and PHYB. Phytochromes can exist in two conformations – the far-red light (P_{FR}) absorbing and the red light (P_R) absorbing forms. In the case of PHYB

in the absence of red light, the inactive red light absorbing (P_R) form dominates and is localized to the cytoplasm. Upon perception of light, the P_R form is converted to the active P_{FR} form and imported into the nucleus as a dimer, whereupon PHYB forms nuclear speckles. PHYA is primarily involved in the perception of far-red light, so R_{FR} is the inactive conformation. Blue light is perceived by the cryptochromes (CRY1 and CRY2), phototropins (PHOT1 and PHOT2) and by the ZTL family. CRY1 is found in the nucleus only in the dark and is normally in the cytoplasm in the light, so its localization is light-regulated. CRY2, on the other hand, is primarily localized to the nucleus independent of light conditions (Lin 2002). Phototropins are localized to the plasma membrane. The ZTL family of proteins includes ZTL, LKP2 and FKF1 – all of which contain a LOV (also known as a PAS) domain, which absorbs blue light (Ito S 2012). They also all contain F-box motifs, which can interact with SKP1-like (ASK) proteins and result in the formation of SCF E3 ubiquitin ligases that aid in the degradation of core clock components like TOC1, CCA1 and LHY. UV-B radiation is detected by the newly discovered UVR8 receptor (Heijde M 2012).

The absence of light signaling as a result of low light conditions or complete darkness as well as the absence of the photoreceptors required for its perception results in hypocotyl elongation, which is especially apparent in etiolated, dark-grown seedlings. This hypocotyl elongation phenotype is usually suppressed at higher light levels, except for in mutants that lack the necessary photoreceptors, such as a *phyB* mutant grown in red light. The absence or the reduction of the ratio of certain wavelengths of light can also have profound effects on plants. One such example is the shade avoidance response, which is caused by the perception of a shift in the red to far-red light ratio to a value less

than 1 (Lorrain S 2008). In nature, this would be caused by the absorption of red light and reflection of far-red light by neighboring plants, thereby reducing the red to far-red light ratio detected by the plant. The plant responds by elongating its hypocotyl and petioles, increasing its leaf angle and in the long run results in early flowering.

Light signaling regulates gene expression

Some photoreceptors are involved in the regulation of gene expression. CRY2 and PHYB have been implicated in the decondensation of chromatin under low light conditions (van Zanten M 2010; van Zanten M 2012). Other photoreceptors have been shown to associate with or otherwise indirectly affect transcription factors. Transcription factor levels can also be modified and post-transcriptionally targeted for degradation following or in the absence of light signaling. These include phytochrome-interacting factors (PIFs), HY5 and HYH as well as homeodomain proteins like ATHB2. In the dark, the E3 ubiquitin ligase COP1 mediates the degradation of transcription factors like HY5 and HFR1 (Jang IC 2005; Yang J 2005; Lorrain S 2006). Consequently, *cop1* mutants have a constitutive photomorphogenic phenotype, meaning that they look like white light grown seedlings in the dark, except for the lack of chlorophyll. COP1 is no longer active when plants are exposed to light, mostly through interactions with the cryptochromes. Phytochromes have also been shown to interact with COP1. PIFs and the related PIL (PIF-like) proteins are bHLH transcription factors that regulate gene expression downstream of photoreceptors, mainly phytochromes but also cryptochromes (Lorrain S 2006). Some of the PIFs are stable in the dark and then degraded following prolonged exposure to light (Castillon A 2009). PIF4 and PIF5 have been implicated in the

activation of ATHB2 as well as hypocotyl elongation in red or blue light (Kunihiro A 2010; Kunihiro A 2011).

Protein degradation mechanisms

One method of down-regulation of mRNA abundance is through microRNA silencing of gene transcripts. miRNAs are processed in plants by a complex of proteins that includes SERRATE, DCL1 (Dicer-like 1) and HYL1 (HYponastic Leaves 1, also known as DRB1, dsRNA binding protein) and requires the cap-binding complex (Fang Y 2007; Montgomery TA 2008; Manavella PA 2012). Proteins can be degraded by the proteasome following ubiquitination by E3 ligases. This can be triggered by phosphorylation or a change in localization that makes the protein more accessible to E3 ligases. Another pathway for altering gene expression is by siRNA-dependent DNA methylation. This process can be dependent or independent of RNA-dependent RNA polymerase 2 (RDR2), which copies ssRNA precursors generated by RNA polymerase IV into dsRNA (Pontier D 2012).

Characterization of TZP

TZP (Tandem Zinc knuckle/PLUS3 domain) is the product of a single copy gene (At5g43630) in *Arabidopsis thaliana* that has orthologs in many other plant species (Loudet O 2008). The gene was discovered using quantitative trait loci (QTL) to compare recombinant heterogeneous inbred family (rHIF) lines between the Sha and Bay-0 ecotypes. The blue light hypocotyl elongation response was used to fine map the resulting quantitative trait loci (QTL). The Bay-0 ecotype was shown to have a premature stop codon in the gene locus that encodes the TZP protein, which has a molecular weight of ~90 kDa. The structure of TZP consists of two tandem zinc knuckle ($CX_2CX_4HX_4C$)

domains and a PLUS3 domain, both on the C-terminal half of the protein. This combination is unique to TZP in *Arabidopsis thaliana*. Zinc knuckle domains are important for interactions with other proteins as well as with RNA and ssDNA (Garrey SM 2006). The PLUS3 domain of RTF1 has also been implicated in nucleic acid binding in yeast via its three conserved positive amino acids as well as in protein-protein interactions (de Jong RN 2008). TZP is found in most plant tissues and is localized primarily in the nucleus, where it forms small punctate structures (nuclear bodies or speckles) in white light. Its localization to the nucleus coupled with its domains suggested a potential role in transcriptional regulation or chromatin remodeling.

TZP is involved in the regulation of morning-specific growth and its mRNA expression peaks around dawn as a result of post-transcriptional regulation by the circadian clock. The overexpression of TZP (*TZP-OX*) results in significantly longer hypocotyls in blue light and increased growth overall. This hypocotyl elongation phenotype is specific to blue or white light, with the loss or gain of full-length TZP having no obvious effects in the dark or in red or far-red light. TZP acts downstream of photoreceptors and the circadian clock, since its expression is disrupted in the circadian clock mutants *lhy* and *elf3*, but the expression of core clock components like GI and CCA1 are not affected when TZP is overexpressed. The effect is post-transcriptional due to the continued cycling of TZP despite overexpression under a constitutively-active promoter.

Overexpression of TZP results in enhanced expression of *HFR1*, *HAT4* (Figure 1) and *IAA1* as well as significantly reduced expression of *NERD* (SWI/PLUS). HFR1 (long Hypocotyl in Far-Red light) is a bHLH transcription factor required for both

phytochrome A- and cryptochrome 1-mediated light signaling (Fairchild CD 2000; Zhang XN 2008). It is unstable in the dark but has high transcript levels in continuous far-red light. IAA1 is a transcription factor in the Aux/IAA gene family that is rapidly upregulated in response to auxin and has been implicated in the control of hypocotyl growth (Colón-Carmona A 2000; Yang X 2004; Meng X 2013). HAT4 (also known as ATHB2) is a homeobox transcription factor with a leucine zipper motif that is expressed in all tissues, with higher levels in the dark or in far-red light (Carabelli M 1993). NERD (NEeded for RDR2-independent DNA methylation) is involved in siRNA-dependent transcriptional gene silencing and associates with AGO2, but does not require RNA-dependent RNA polymerase 2 (RDR2) (Pontier D 2012). Misregulated genes in *TZP-OX* also had an overrepresentation of morning-specific response elements like the morning (CCACA), G-box (CACGTG) and HUD (Hormone Up at Dawn, CACATG) elements in their promoters, further validating that TZP is involved in regulation of morning-specific gene expression (Loudet O 2008; Michael TP 2008).

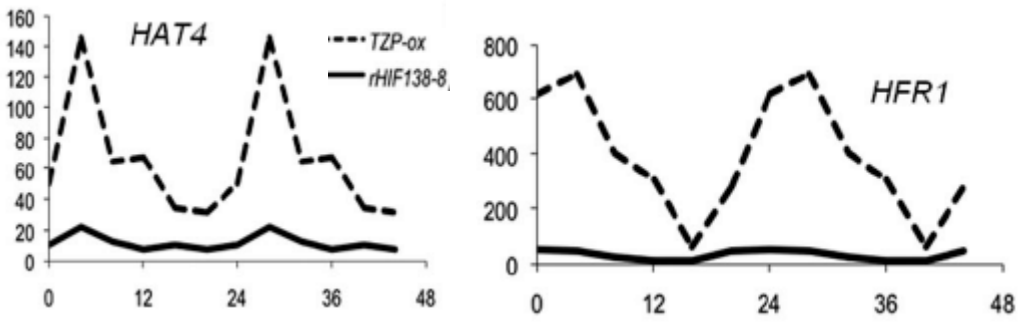


Figure 1 – Enhanced expression of *HAT4* and *HFR1* when TZP is overexpressed (Loudet et al. 2008, figure S9C and S9I)

Proteins that interact with TZP

Some work has previously been done to identify proteins that interact with TZP via yeast two hybrid and pull-down assays. TZP interacts very strongly with the

transcription factor ATHB23 (At5g39760) *in vivo* and in yeast two hybrid assays, an interaction that requires the PLUS3 domains of TZP and the zinc finger domains of ATHB23. ATHB23 is a member of the zinc-finger homeodomain (ZF-HD) protein family. It can form dimers with itself or with other ZF-HD proteins via the conserved zinc finger domains at the N-terminus (Tan QK 2006). The homeodomain is a 60-amino acid DNA binding domain.

TZP also interacts with PHYB in yeast two-hybrid assays as well as in coimmunoprecipitation assays, an interaction that is dependent on the presence of the N-terminus of TZP. TZP and PHYB colocalize *in vivo*, but this depends on the presence of PHYB and at least the N-terminus of TZP.

TZP also copurifies with important circadian clock components and colocalizes with RAD51, the blue light receptor CRY2, the phytochrome signaling regulator SPA1 and the phytochrome-interacting factors PIF4 and PIF5. RAD51 is involved in the pairing of homologous chromosomes and in DNA repair mechanisms, including the repair of double-stranded breaks (Doutriaux MP 1998; Osakabe K 2002). Its expression is induced by gamma, UV and other forms of radiation.

The colocalization and interactions of TZP with ATHB23, CRY2, PHYB, PIF4 and PIF5 as well as the presence of the PLUS3 and zinc knuckle domains indicates potential interactions with proteins involved in gene regulation, possibly through interactions (direct or indirect) with other transcription factors, photoreceptors or with proteins involved in gene silencing – none of which had been investigated yet.

Light-dependent nuclear body formation

Some work has also been done on the localization of TZP. It appears to only localize to nuclear bodies in red or white light, but not blue or far-red light (Figure 2). When transferred from white to blue light, TZP nuclear bodies start to disappear as the protein assumes a more uniform nuclear distribution, so nuclear body formation is dynamic. Localization to nuclear bodies is dependent on the interaction of TZP with PHYB, since TZP does not form nuclear bodies under any light conditions in *phyB* mutants.

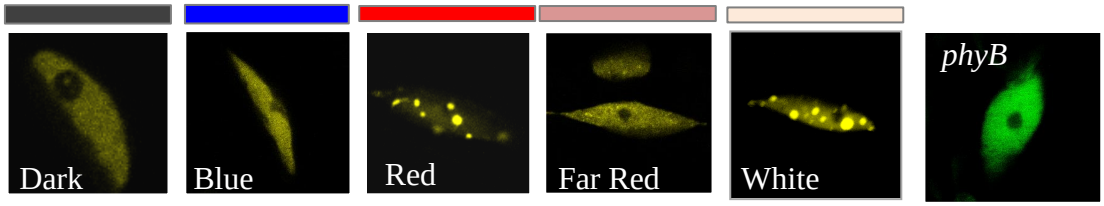


Figure 2 – Nuclear body formation under dark, blue, red, far-red or white light conditions. No nuclear bodies form in *phyB* mutant. (courtesy of Eirini Kaiserli)

Analysis of truncations

Truncated forms of TZP have also been used to determine what domains are required for its function and for its localization (Figure 3). The N-terminus alone (Nt) was still able to localize to nuclear bodies, but there was no significant increase in hypocotyl length in blue light compared to Col. Truncated forms that contained only the PLUS3 domain or the PLUS3 domain with the adjacent tandem zinc knuckle domains (ZFPL) were not able to localize to nuclear bodies and had a shorter hypocotyl than Col in blue light (dominant negative phenotype). The localization and phenotype of these truncations has been investigated, but not the inherent protein stability of each truncation.

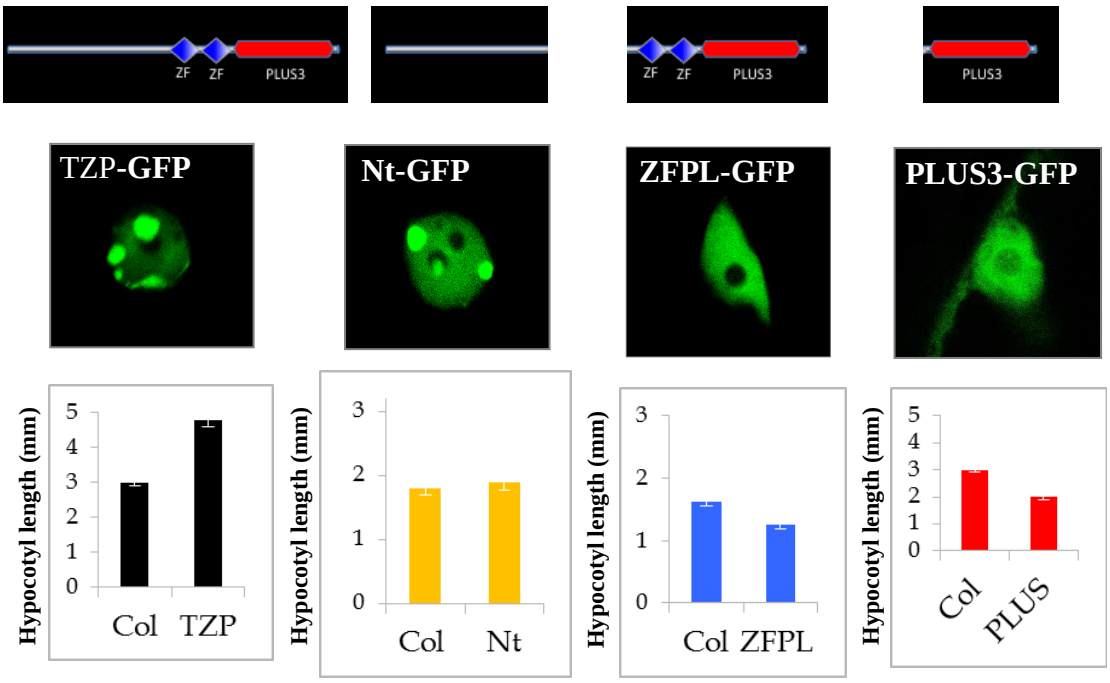


Figure 3 – Diagrams, intercellular localization and hypocotyl length of *TZP-OX* compared to various truncations (N-terminus alone, PLUS3 domain with zinc knuckles or PLUS3 alone – all tagged with GFP for visualization, courtesy of Eirini Kaiserli)

EAR motifs and transcriptional repression

TZP contains an EAR motif in the PLUS3 domain (Figure 4), which is conserved in all accessions and in the closely related species *Arabidopsis lyrata* as well as some other plant species including *Populus trichocarpa*, *Vitis vinifera* (grape) and *Glycine max* (soybean) (Loudet O 2008). EAR stands for ERF (ethylene-responsive element binding factor)-associated amphiphilic repression (Ohta M 2001; Kagale S 2010; Kagale S 2011). Proteins that contain these motifs can influence the epigenetic regulation of gene expression by recruiting chromatin-remodeling factors, which results in active transcriptional repression. EAR motif proteins have been implicated in the regulation of salt, drought and cold stress (Ciftci-Yilmaz S 2007; Dong CJ 2010; Pan IC 2010). Several corepressors have been shown to be involved in this transcriptional repression – namely TPL, SAP18 and SIN3. SAP18 (SIN3-associated polypeptide of 18 kDa) has been shown

to interact directly with EAR-motif-containing proteins, an interaction that is lost when the leucines in EAR motif consensus (LxLxL) sequence are mutated to alanines (Hill K 2008; Szemenyei H 2008; Pauwels L 2010; Shyu C 2012). HDA19, a histone deacetylase, has also been identified as a chromatin-remodeling factor involved in this process. The importance of the EAR motif of TZP had not been investigated.

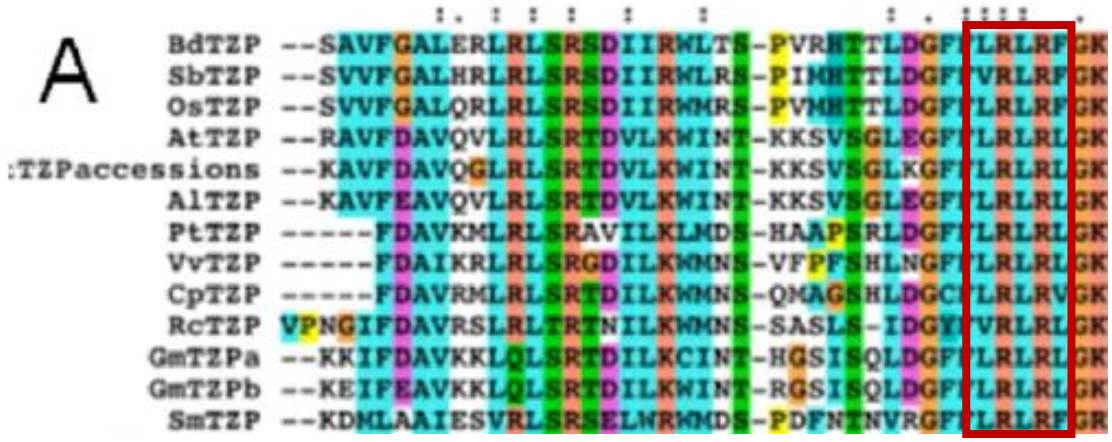


Figure 4 – EAR motif of TZP in PLUS3 domain and alignment with TZP orthologs from other plant species (adapted from Loudet et al. 2008, figure S3A)

In this thesis, I will present some results showing how the protein stability of TZP and its truncations changes in response to light, what effect different mutant backgrounds have on this stability as well as some new interactions with TZP in yeast, with the intent of figuring out more about the function of TZP. Given that TZP has a phenotype in blue light but colocalizes with PHYB in nuclear bodies in red light, one objective of my project was to determine what light conditions and light signaling components affect its protein levels as well as to see what domains of TZP are responsible for these changes in protein abundance. In overexpression lines with constitutive expression of TZP at the transcript level, protein levels preferentially accumulated in blue light. This accumulation was shown to be continuous and the low levels in the dark were due in part to

degradation via the proteasome. The protein levels of the truncations differed in their responsiveness to blue light treatment. Some mutants in the blue light signaling pathways also showed differences in TZP levels, although whether this is due to regulation at the level of the transcript or the protein remains to be determined. Yeast-two hybrid analysis was used for preliminary analysis of potential interactions of TZP with proteins that are involved in the regulation of gene expression. Positive interactions were seen with proteins involved in active transcriptional repression that are known to associate with EAR motifs (such as the one in the PLUS3 domain of TZP) as well as with proteins involved in miRNA processing and with the blue light receptor LKP2, a member of the Zeitlupe family that have been shown to recruit E3 ligases.

Acknowledgments

Some of Eirini's work, as of yet unpublished, is included in this thesis, including the work previously done on TZP discussed in the introduction (figures 2 and 3).

Chapter 1 – Protein Levels

TZP has a hypocotyl elongation phenotype in blue light but forms nuclear bodies only in red or white light. The first step was to see how different wavelengths of light and different durations of exposure to light affected protein levels in lines that overexpressed TZP. Since TZP is overexpressed under the 35S promoter in these lines, transcription should be maintained at a constant high level. Therefore, it would be possible to observe changes at the protein level due to changes in stability, not transcript levels. Any changes in stability were then further investigated using MG132 treatment to look at how the absence of proteasome degradation would affect protein levels, since proteins like CIB1 and HFR1 that have light-induced stability are usually degraded by the proteasome in the dark (Yang J 2005; Liu H 2013). The stability and pattern of accumulation of the truncations was then investigated to see the inherent stability of each domain under blue light conditions. Given the blue light-associated phenotype of TZP and its interaction with PHYB (a red light photoreceptor), various mutants in the red and blue light signaling pathways were then investigated to see what protein interactions, both direct and indirect, affected TZP protein levels in blue light.

1.1 – Protein levels accumulate after exposure to blue light

Based on previous data that demonstrated a phenotype for lines overexpressing TZP in blue light, protein levels were compared between dark-grown etiolated *TZP-OX* seedlings exposed to blue, red and far-red light. Compared to the dark controls, TZP protein levels accumulated most significantly following blue light treatment (Figure 5). Coilin is a protein that also forms punctate structures in the nucleus – namely Cajal bodies – regardless of light conditions (Collier S 2006). The levels of Coilin did not

change between the light conditions. Dark-adapted white light grown seedlings also had accumulation when treated with blue light (data not shown), but the effect was not as dramatic as for dark-grown etiolated seedlings, so dark-grown seedlings were used for consistency in all future experiments.

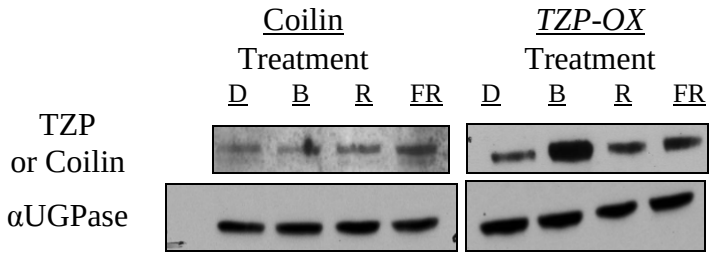


Figure 5 – Accumulation of TZP protein (anti-GFP) in 4-day old dark-grown seedlings either kept in the dark (D) or moved to blue (B), red (R) or far-red (FR) light for 4 hours; Coilin (anti-DsRed) used as negative control, UGPase used as loading control

Since TZP accumulated most significantly in blue light, a more in-depth time course in blue light was examined with *TZP-OX* seedlings. This time course showed continuous accumulation of TZP protein levels throughout the course of the blue light treatment (Figure 6). The accumulation became more pronounced after 4 hours of blue light treatment, but continued at the 8 and 24-hour time points. The levels started off low in the dark despite constitutive expression with the 35S promoter, suggesting that TZP is constantly degraded in the dark and then stabilized in blue light.

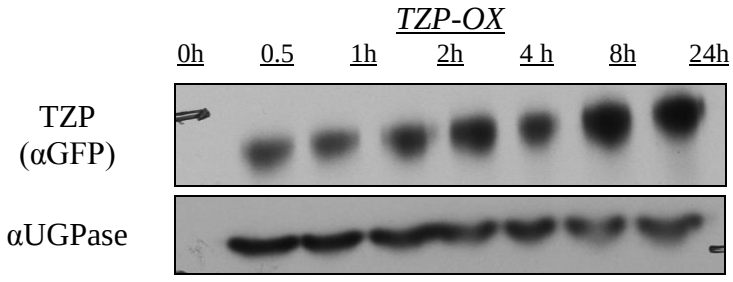


Figure 6 – TZP protein continues to accumulate for the duration of blue light exposure (0, 0.5, 1, 2, 4, 8 and 24 hour treatment). Anti-GFP used for TZP (tagged overexpression lines) and anti-UGPase used as loading control.

1.2 – MG132 treatment reveals proteasome dependent degradation of TZP in dark

MG132 treatment was implemented to examine whether the stability of the protein is a factor in its accumulation. MG132 treatment reduces the degradation of ubiquitinated proteins by the proteasome. With this treatment, TZP levels were constitutively higher – especially in the dark (Figure 7) – suggesting that TZP is normally targeted for degradation by the proteasome in the dark, based on its low levels, then stabilized following exposure to blue light, either by a change in localization or as a result of interactions with other proteins that protect it from degradation. It could also be the case that the proteins that ubiquitinate TZP in the dark are degraded or inactivated in blue light, possibly through phosphorylation or dissociation of required cofactors. Recently, ZTL and LKP2 have been linked to the rapid, enhanced stability of CIB1 in blue light by preventing CIB1 degradation by the proteasome that normally occurs in the dark (Liu H 2013). This parallels the regulation of TZP protein levels, so it is possible that the Zeitlupe proteins play a role in this case as well. In the case of CIB1, the loss of either of these Zeitlupe proteins prevents protein accumulation in blue light and CIB1 is still degraded in the dark, so it would be useful to see how TZP protein levels are affected in the Zeitlupe mutants.

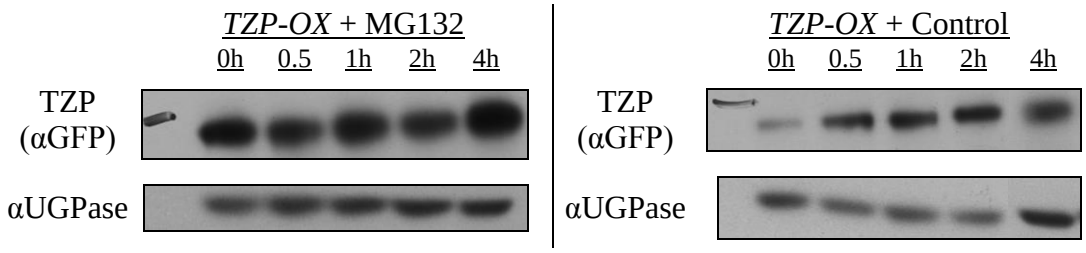


Figure 7 – TZP protein levels in overexpression lines with MG132 treatment or control. 4 day old dark-grown seedlings transferred to blue light for 0.5, 1, 2 or 4 hours with continuous soaking in MG132 (50 µM) or control for 5 hours (started for all seedlings one hour prior to 4 hour blue light treatment)

1.3 – Regulation of protein abundance in TZP truncations

The next step was to look at how the levels of the various truncations responded to blue light treatment, since the localization and phenotype of the mutants varied. All of the truncations were overexpressed in the Col background using the 35S promoter with a GFP tag for localization and western blot analysis. The PLUS3 domain with GFP was about 45 kDa in size, the N-terminus with GFP was about 95 kDa in size and the ZF-PLUS, which contained both of the tandem zinc knuckles and the PLUS domain with GFP, was about 55 kDa. PLUS3 domain on its own had much higher levels in the dark and no significant accumulation in blue light (Figure 8). The absence of the N-terminus appeared to prevent degradation in the dark, possibly due to the lack of an interaction with PHYB. The lack of accumulation in blue light could be attributed to the lack of the zinc knuckles and/or the N-terminus or just due to constitutively high levels to start with. The ZF-PLUS and the N-terminus both showed accumulation in blue light and lower levels in the dark. The N-terminus had delayed accumulation (at 8 hours not 4 hours), while the ZF-PLUS had a normal accumulation pattern. The truncation with just the N-terminus lacks both the PLUS3 domain and the zinc knuckles, which are the only two predicted domains in TZP, so it may be unstructured and therefore less stable in general. The zinc knuckles appear to result in decreased stability in the dark and normal accumulation in blue light, both of which are absent in the truncation with just the PLUS3 domain. This suggests that the zinc knuckles are responsible for the interactions with other proteins that give TZP its characteristic pattern of protein accumulation. However, it is important to note that none of the truncations displays the typical blue light hypocotyl elongation phenotype observed only when full-length TZP is overexpressed,

suggesting that it is the unique combination of the PLUS3 domain and the zinc-knuckles as well as some unique sequences in the N-terminus that contribute to TZP's function.

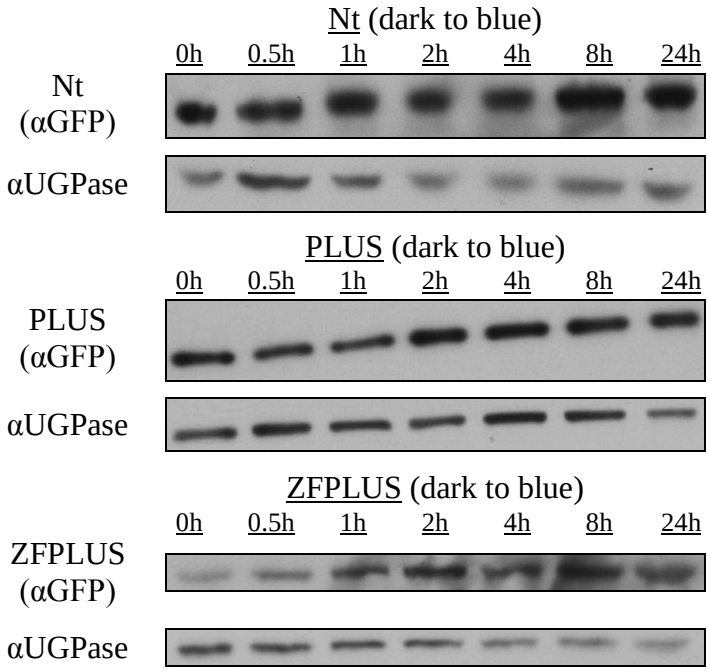


Figure 8 – Protein levels of truncations in respond to blue light exposure (0, 0.5, 1, 2, 4, 8 and 24 hour treatment). Anti-GFP (tagged lines) for used for truncations and anti-UGPase used as a loading control.

1.4 – Investigation of TZP protein levels in the absence of light signaling components

In order to see know different proteins influence TZP stability and accumulation in blue light, TZP protein levels were examined using a native antibody in wild-type and in various mutant backgrounds (Figure 9). Since TZP is under its native promoter in these lines, one cannot distinguish between increased transcript levels and increased stability at the protein levels if protein levels are higher than normal. In the *TZP-OX* lines, the 35S promoter ensured constitutively-high mRNA expression, so any changes in protein levels were due to increased stability at the protein level.

The endogenous TZP protein levels in Col follow the same pattern as in the overexpression lines. In the *pif4-pif5* double mutant background, TZP protein levels were

higher in the dark and then decreased following blue light treatment, suggesting that PIF4 and PIF5 play an important role in the accumulation of TZP in blue light and its down-regulation (at mRNA level) or degradation (at protein level) in the dark. The triple mutant in CRY1, CRY2 and PHOT1 (*C1C2P1*) showed a similar phenotype to the *pif4-pif5* double mutant in that TZP protein levels in the dark were higher than normal and TZP levels decreased in blue light. Lack of phytochrome A, phytochrome B and cryptochrome 1 (single mutant lines) did not affect TZP degradation in the dark or accumulation in blue light. This was also seen with the GFP antibody in lines overexpressing TZP in *phyA* and *phyB* mutant backgrounds (data not shown). Also, lines expressing a dominant, gain-of-function, light-independent and constitutively-active PHYB (Y276H) had a similar accumulation pattern to the *phyB* mutant, which further supports the idea the interaction of the N-terminus of TZP with PHYB is not required for TZP stability. The *phyA-phyB* double mutant also showed accumulation in blue light, indicating that there is no compensatory effect by the other phytochrome in either of the single mutants. The *cry2* single mutant did show delayed accumulation in blue light, which could be explained with the fact that CRY2 has been shown to colocalize with TZP and this may help influence its increased levels in blue light. In the *zlf* mutant, which lacks all three members of the Zeitlupe protein family (ZTL, LKP2 and FKF1), the levels of TZP are higher in the dark and accumulate rapidly following blue light treatment. Members of the Zeitlupe family are involved in blue light dependent degradation of core clock components like TOC1 and PRR5, so this might explain the increased stability of TZP in the triple mutant. Conversely, ZTL and LKP2 have also been implicated in the blue light induced stability of CIB1, which is normally degraded in the dark. In this case, CIB1

levels were lower in blue light in the *ztl* and *lkp2* mutants (Liu H 2013). This differs from what happens with TZP in the triple mutant, where the Zeitlupe family seems to be involved in the degradation of TZP but not its increased levels in blue light.

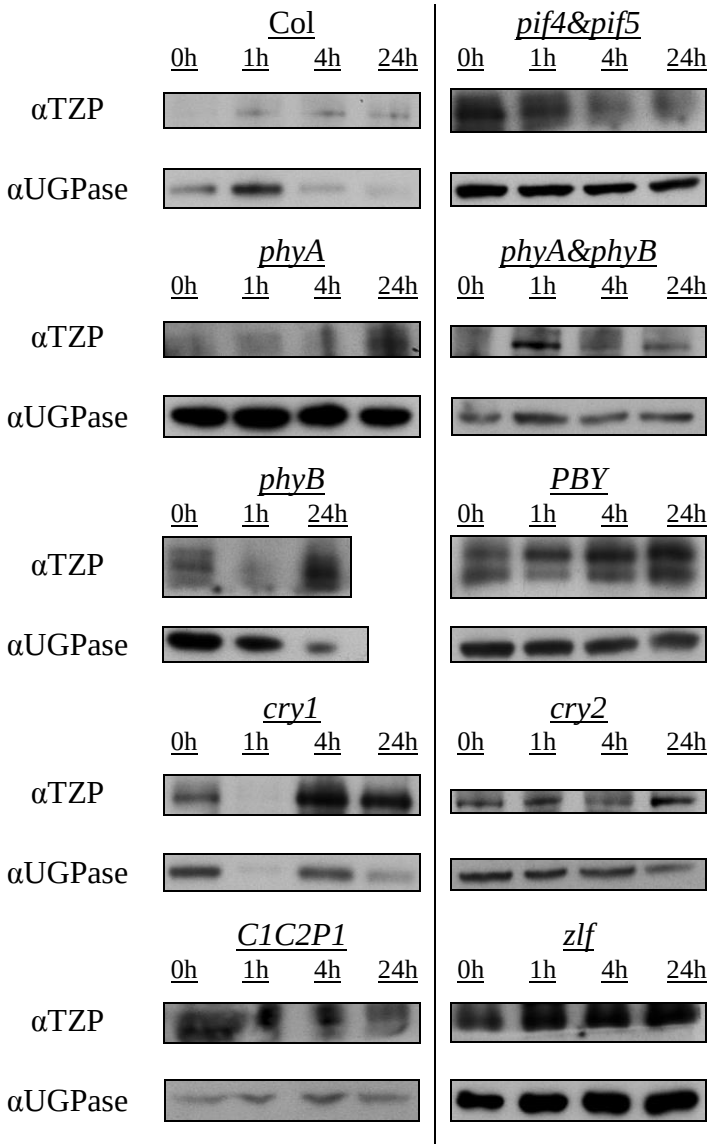


Figure 9 – TZP protein levels using native TZP antibody in Col (wild-type) as well as the *phyA, phyB, PBY, phyA&phyB, pif4&pif5, cry1, cry2, C1C2P1* and *zlf* mutant backgrounds

Chapter 2 – Testing for TZP interactions using Yeast Two Hybrid

The principle behind the yeast two-hybrid assay is that two fusion proteins are co-expressed, one of which is fused to the GAL4 activation domain (AD) and the other is fused to the GAL4DNA-binding domain (DBD). The plasmids for these fusion proteins also contain genes that allow the yeast to synthesize leucine and tryptophan, so colonies that express both plasmids would now be able to grow on media that lacks both leucine and tryptophan. Without a positive interaction, yeast colonies that have been selected to possess both plasmids would not be able to grow without histidine in the presence of the inhibitor 3AT. The *HIS3* gene has been inactivated in these yeast strains, but some leaky expression still persists and high enough levels of 3AT in the media would inhibit the growth of colonies without a positive interaction between the two fusion proteins. When the two fusion proteins interact, the GAL4 AD and DBD together can bind the GAL4 element upstream of the *HIS3* gene and recruit RNA polymerase to induce *HIS3* expression, thereby allowing the colonies to grow even with increasing 3AT concentrations. The strength of the interaction is reflected by how much 3AT is required to prevent the growth of colonies on media with no added histidine.

Yeast two hybrid was used to see if TZP interacts with proteins known to be EAR-motif associated corepressors or with proteins involved in gene silencing. Yeast two hybrid allows for the preliminary identification of direct interactions between proteins. Confirming direct interactions *in planta* would require the use of BiFC or other similar approaches, which take longer than first identifying potential interactions in yeast. One drawback of using yeast is that post-transcriptional modifications normally present in plants, including phosphorylation and proper folding, could be absent in yeast. However,

using yeast in most cases ensures that the interactions observed are direct and not an artifact of binding through an intermediate partner, which can occur with colocalization experiments *in planta*.

The potential role of the EAR motif in the PLUS3 domain of TZP was investigated by testing its interactions with known corepressors such as SAP18, SIN3 and TPL. The nucleic acid binding capabilities of the PLUS3 and tandem zinc knuckle domains suggested potential roles for TZP in DNA or RNA binding. Proteins involved in miRNA processing, like SERRATE and SAP18, were intriguing candidates due to their link to the regulation of gene expression. ATHB23 was used as a positive control based on a strong, previously-shown interaction with TZP. Negative controls were used with the corresponding empty vector to test for autoactivation. TZP interacted strongly with SERRATE, TPL, SAP18 and LKP2 as well as with HDA19 and HYL1.

2.1 – Investigating potential interactions with known transcriptional co-represssors

TZP interacted with two known corepressors (TPL and SAP18) associated with EAR motif-dependent, active transcriptional repression (figure 10 and 13). In this case, just the N-terminus of TPL (amino acids 1-173) was used for yeast two hybrid, since this is the domain shown to be required for interactions with EAR motifs (Szemenyei H 2008). The TZP EAR mutant that lacks the leucines in the EAR motif (ARARA), which are critical for its function, had a much weaker interaction with these two proteins (figure 13 and 14). The TZP EAR mutant that lacks the arginines (LALAL), which are not part of the consensus sequence for the EAR motif, was still capable of interacting with SAP18, while the PLUS3 domain alone was not sufficient for interaction with SAP18 (figure 12). SIN3, which has not been shown to interact directly with EAR motifs, failed

to interact with TZP (figure 10). HDA19, a chromatin deacetylase shown to be recruited by EAR motif associated corepressors, also interacted (but weakly) with full-length TZP or with just the N-terminus, but not with just the PLUS3 domain (figure 10, 15 and data not shown). It also failed to interact with the TZP EAR mutant (figure 14).

2.2 – TZP interactions with microRNA processing components

The interaction with SERRATE as well as with HYL1 (figure 11), both of which are involved in miRNA processing, indicates a possible role for TZP in gene silencing. The interaction of SERRATE and TZP was not dependent on the EAR motif, since the TZP mutant lacking the arginines in the EAR motif still interacted with SERRATE and mutating the leucines did not prevent either interaction (figure 12 and 14). Also, the PLUS3 domain of TZP was not sufficient for the interaction with either protein, but both proteins were able to interact with just the N-terminus (figure 12 and 15).

2.3 – TZP interactions with blue light signaling components

TZP also interacted with LKP2 (figure 11), a member of the Zeitlupe protein family involved in blue light signaling as well as photoperiodic control of flowering and circadian clock. LKP2 contains an F-box motif, which can interact with SKP1-like (ASK) proteins, resulting in the formation of E3 ubiquitin ligases and the degradation of core clock components like TOC1 and PRR5 (Schultz TF 2001). The overexpression of LKP2 also results in hypocotyl elongation under blue light conditions, just like the blue light dependent response of lines overexpressing TZP.

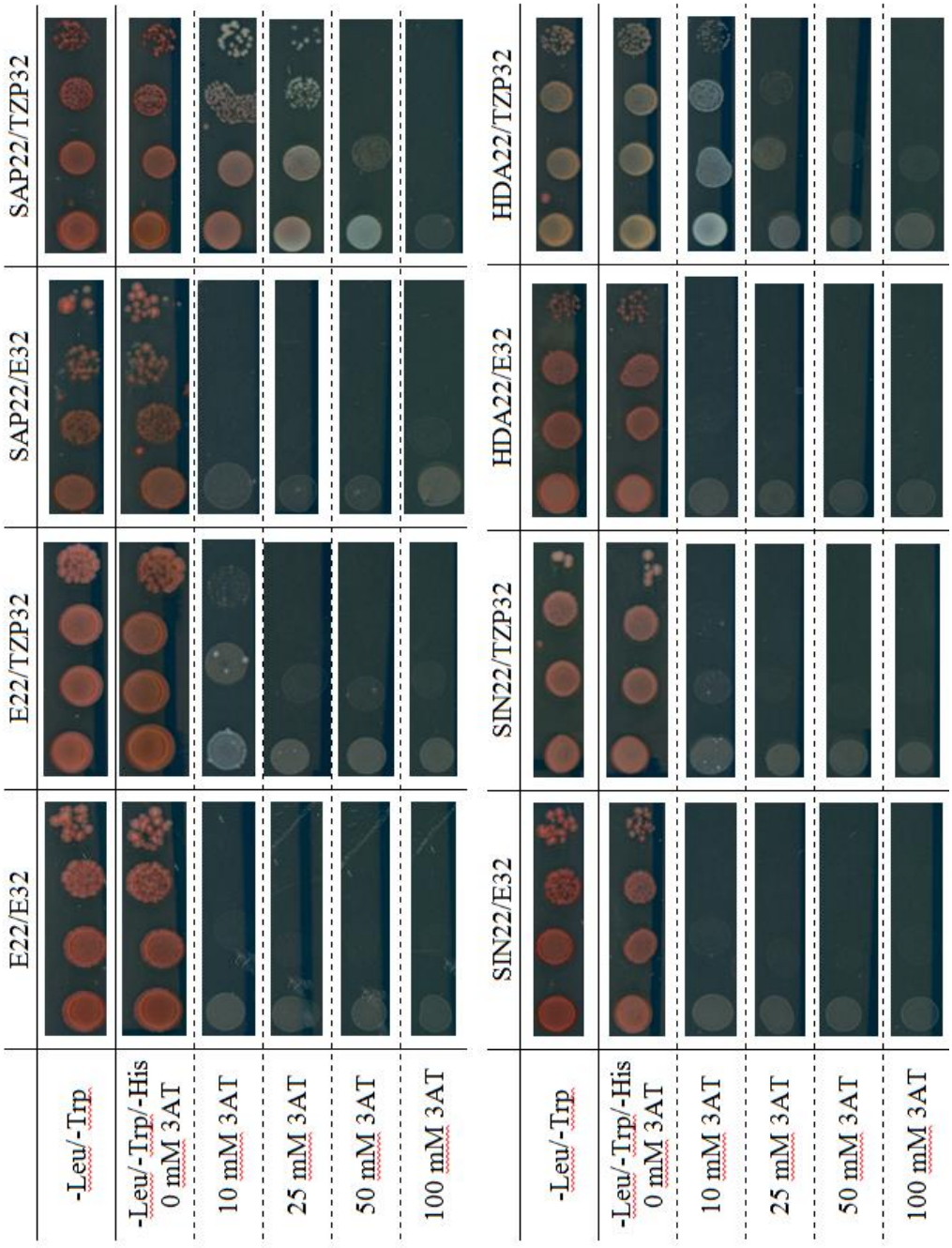


Figure 10 – Positive TZP interaction with SAP18 and HDA19 (both in pDEST22) but not with SIN3. Negative controls are included to show the absence of autoactivation between TZP-pDEST32, SAP18-pDEST22 and HDA19-pDEST22 and their corresponding empty vector.

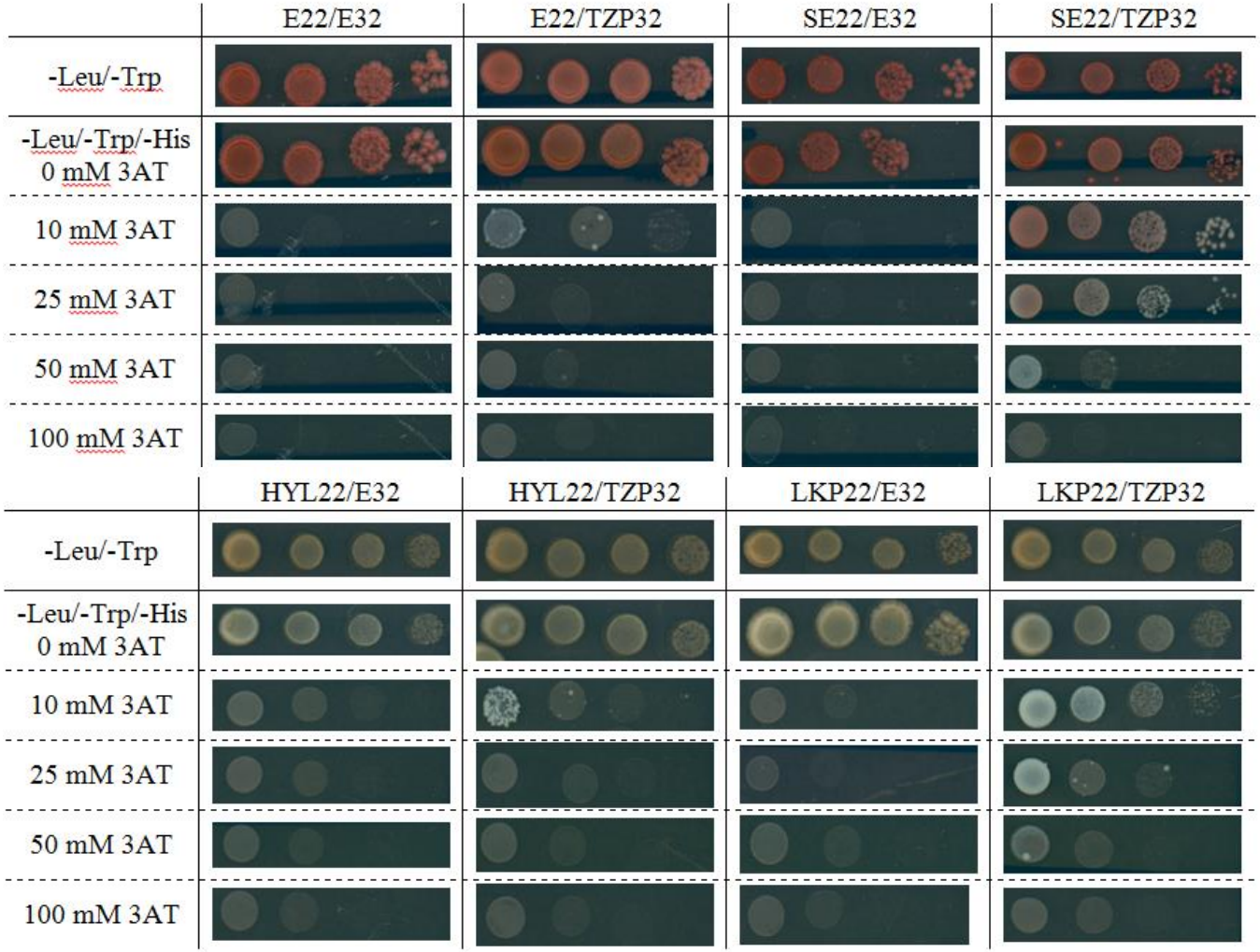


Figure 11 – Positive TZP interaction with SERRATE, HYL1 and LKP2 (all in pDEST22). Negative controls are included to show the absence of autoactivation between TZP-pDEST32, SE-pDEST22, HYL1-pDEST22 and LKP2-pDEST22 and their corresponding empty vector.

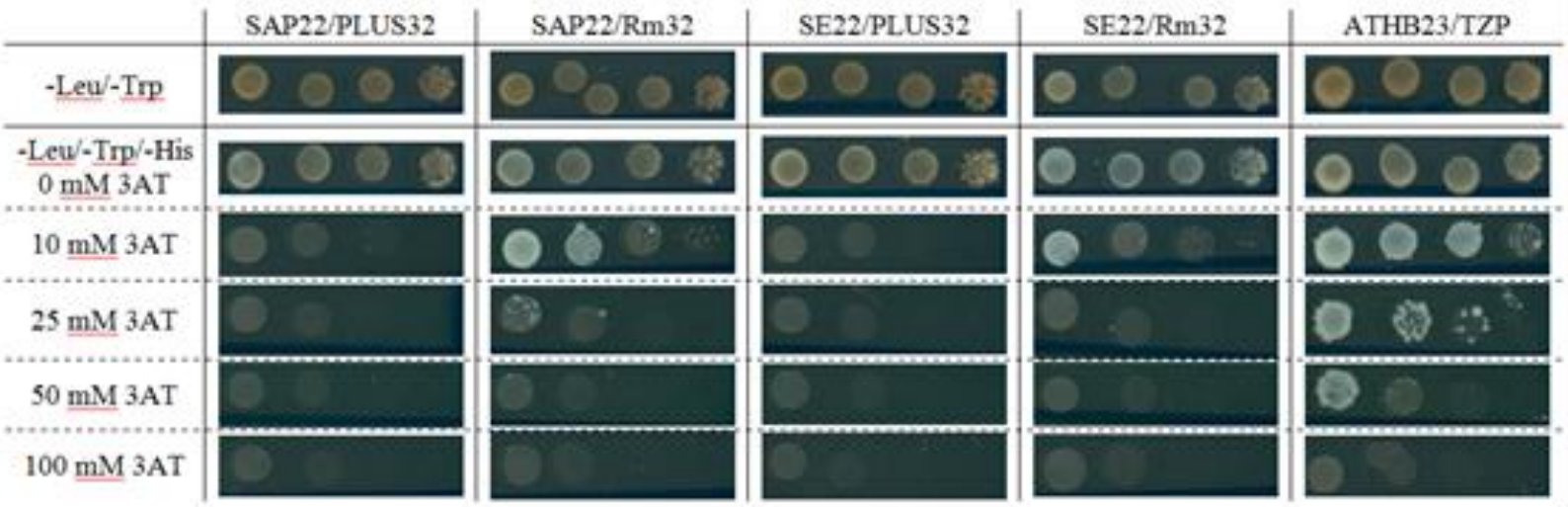


Figure 12 – Positive interaction of SERRATE and SAP18 (both in pDEST22) with a TZP mutant that lacks the arginines in the EAR motif (LRLRL→LALAL). As a positive control, ATHB23-pDEST22 is shown here interacting with TZP-pDEST32. The PLUS3 domain alone is not sufficient for interactions with SERRATE or SAP18.

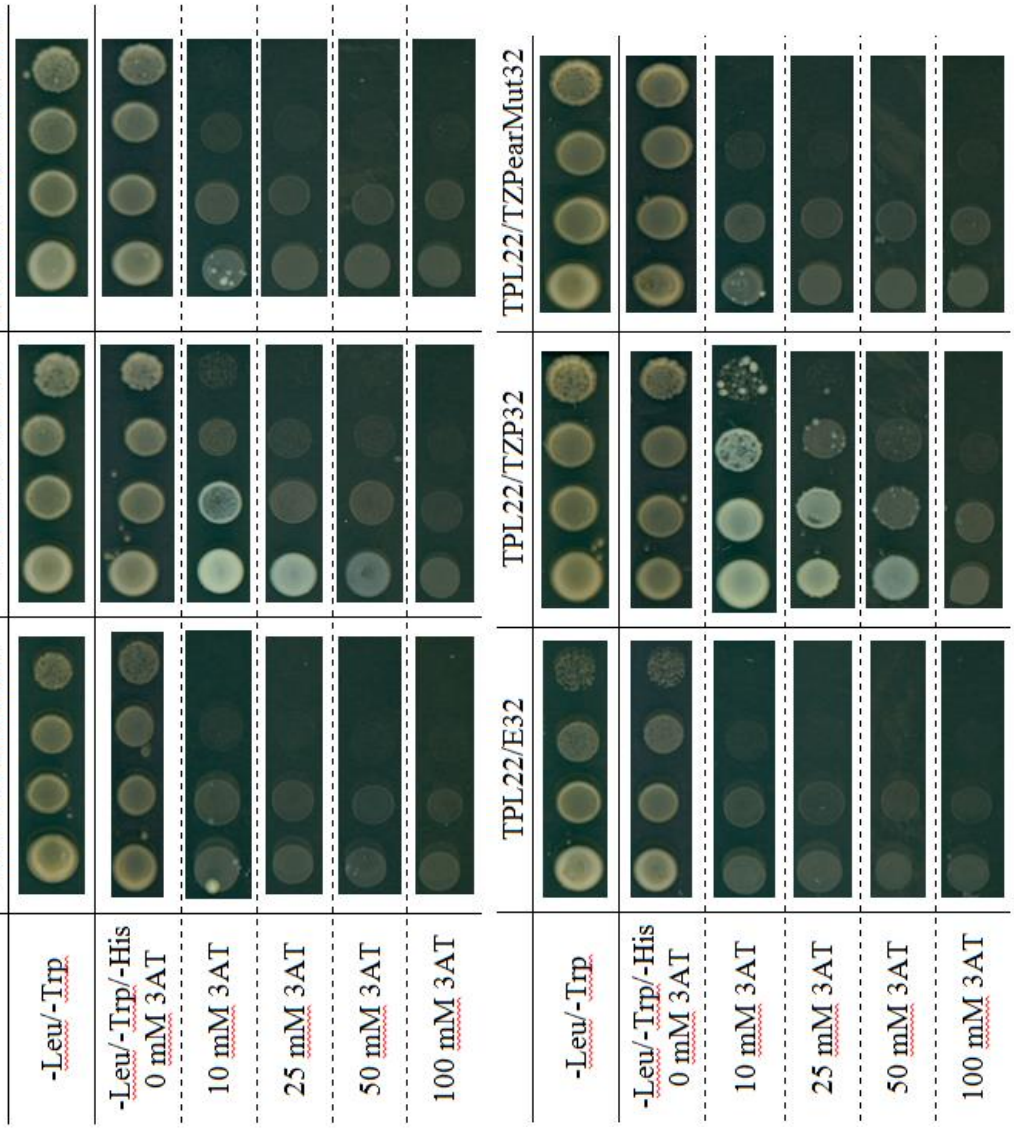


Figure 13 – Positive TZP interaction with TPL and ATHB23 (both in pDEST22), but reduced interaction of TZP EAR mutant (LRLRL→ARARA) with both proteins.

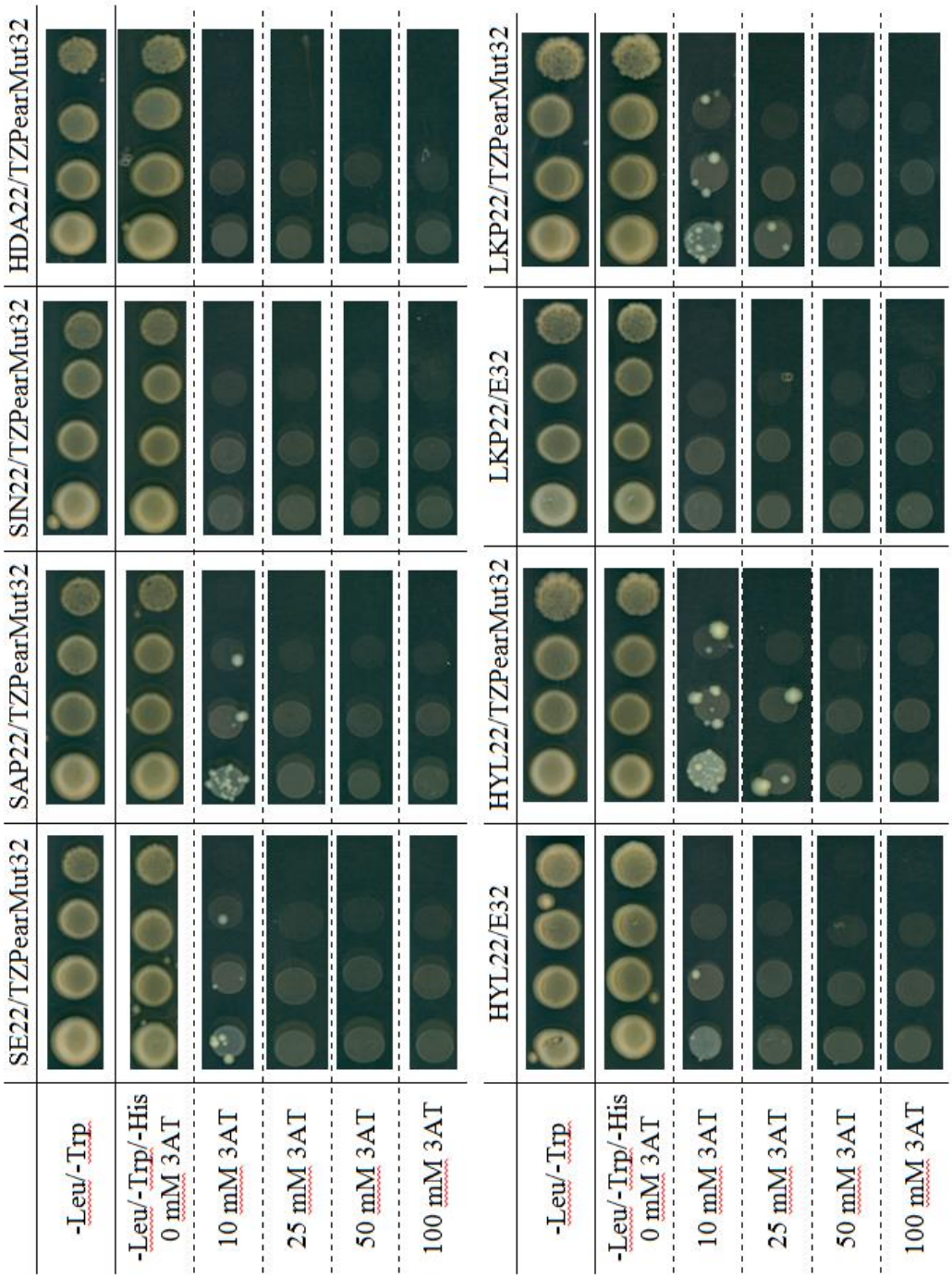


Figure 14 – Interactions of SERRATE, SAP18, SIN3, HDA19, HYL1 and LKP2 with TZP EAR mutant (LRLRL→ARARA). Positive interactions only with SERRATE, SAP18, HYL1 and LKP2. The interaction with is weaker than with wild-type TZP

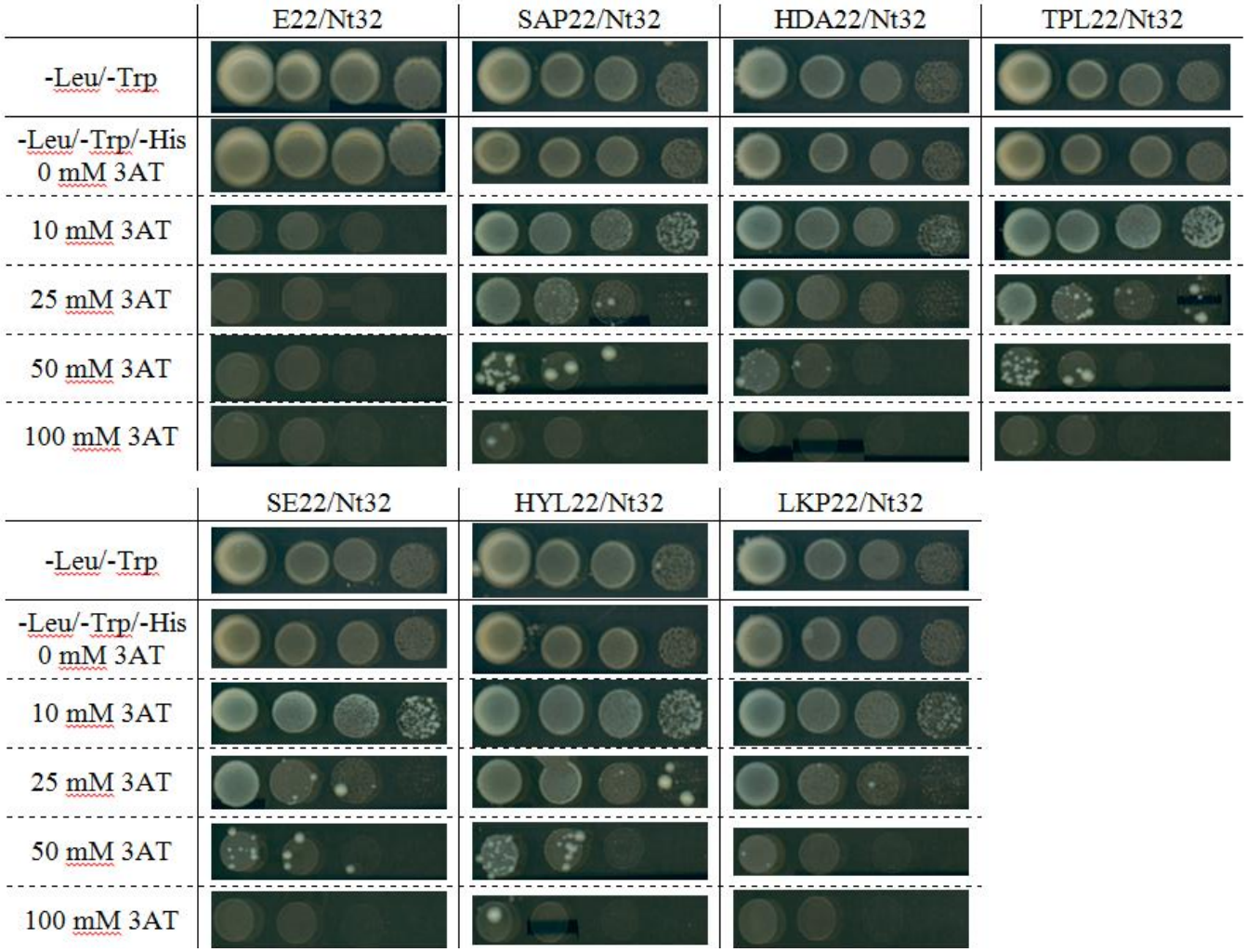


Figure 15 – Positive interactions seen between Nt-pDEST32 and SAP18, HDA19, TPL, SERRATE, HYL1 and LKP2. Negative control included with Nt-pDEST32 and empty pDEST22 vector to show lack of autoactivation.

Discussion

TZP protein is stabilized by blue light

TZP was previously known to be involved in morning-specific growth, downstream of photoreceptors and the clock, with a blue light dependent phenotype (Loudet O 2008). Here, I showed that TZP protein levels accumulate most significantly following blue light treatment, due in part to the increased stability following exposure to and in the presence of blue light. The low levels in the dark are most likely due to degradation by the proteasome, although low transcript levels may also play a role. MG132 treatment demonstrated that TZP is indeed normally degraded in the dark, since levels were higher in the dark when the proteasome was inhibited. This suggests an interaction between TZP and an E3 ligase as well as possible sumoylation or ubiquitination – theories that have to be investigated further. CIB1, which is usually degraded in the dark like TZP, has been shown to be stabilized in the presence of blue light via protection from degradation by ZTL and LKP2, two members of the Zeitlupe family (Liu H 2013), so perhaps TZP follows a similar pathway.

Zinc knuckles are important for blue light accumulation

Out of the truncations, only the N-terminus was able to localize to nuclear bodies and its levels still accumulated in response to blue light, although this accumulation peaked later. The PLUS3 domain on its own, as well as with the adjacent tandem zinc knuckle domains, failed to localize to nuclear bodies and had a shorter hypocotyl in blue light compared to Col. The PLUS3 domain on its own was more stable in the dark and lacked the obvious accumulation in response to blue light seen with the full-length protein. When the zinc knuckles are included with the PLUS3 domain, the levels

accumulate normally, even though localization to nuclear bodies is impaired. The hypocotyl elongation phenotype, however, requires the full-length protein. This implies that each part of the protein – the N-terminus with its interaction with PHYB, the PLUS3 domain with its interaction with ATHB23 and the zinc knuckles with their effect on protein stability – plays a role in TZP's function and stability in blue light.

Blue light signaling components are important for typical accumulation pattern

The phytochromes PHYA and PHYB do not appear to regulate the protein accumulation of TZP in blue light, although evidence has shown that TZP can interact directly with PHYB. Some components of the blue light signaling pathway, however, do affect TZP levels, as would be expected of a protein with a blue light dependent phenotype. The lack of both PIF4 and PIF5 adversely affects TZP stability in blue light, but, at the same time, stability in the dark is increased. CRY1 alone does not appear to affect TZP accumulation in blue light, but the loss of CRY2 resulted in delayed accumulation, indicating that CRY2 may play a role in the blue light dependent accumulation of TZP. The *cry1-cry2-phot1* (*C1C2P1*) triple mutant had higher levels in the dark and lower levels in blue light. The involvement of PIF4 and PIF5, both of which are transcription factors, suggests that TZP levels may be regulated (directly or indirectly) by transcription. The triple mutant of the Zeitlupe family, which is involved in blue light signaling, also had higher TZP levels in the dark but, unlike the *pif4-pif5* double mutant and the *cry1-cry2-phot1* triple mutant, accumulation in blue light still occurred. This indicates that the ZTL family of proteins is not directly involved in the pathway that perceives blue light and triggers the accumulation of TZP. This is in

contrast to what happens in the case of CIB1 stabilization in blue light by ZTL and LKP2, where the loss of either Zeitlupe protein results in lower levels in blue light.

TZP interactions in yeast point to role in regulation of gene expression

Yeast two hybrid analysis showed that TZP interacts with some known corepressors through its EAR motif in the PLUS3 domain, suggesting a role for TZP in active transcriptional repression. This interaction is impaired in a mutant form of TZP that lacks with leucines in the EAR motif, but the PLUS3 domain alone was not sufficient for this interaction. This indicates that, although the EAR motif may be important for interactions with these corepressors, the full-length protein is still required. Some preliminary localization experiments using the TZP EAR motif mutant in tobacco revealed that the mutant does not form nuclear bodies (data not shown, courtesy of Eirini Kaiserli), which is interesting given that the EAR motif is in the PLUS3 domain and the N-terminus is required for colocalization with PHYB in nuclear bodies. Coupled with the conservation of the EAR motif in TZP orthologs of some other plant species, these results seems to indicate that the EAR motif is important for TZP's function.

TZP also interacts with SERRATE and HYL1, both of which are involved in miRNA processing, possibly via the N-terminus. This indicates a possible role for TZP in gene silencing. The interaction of TZP with the transcription factor ATHB23, however, indicates a possible role in activation of transcription as well. The interaction with LKP2 may help explain the link to the hypocotyl elongation phenotype in blue light with the overexpression of TZP. It may also provide the link to both light signaling and the circadian clock, since LKP2 is involved in both processes. All of these yeast interactions would have to first be confirmed first *in planta*.

Acknowledgments

Some of Eirini's work, as of yet unpublished, is included in this thesis, including the work done on the localization and expression of the EAR mutant of TZP mentioned in this discussion.

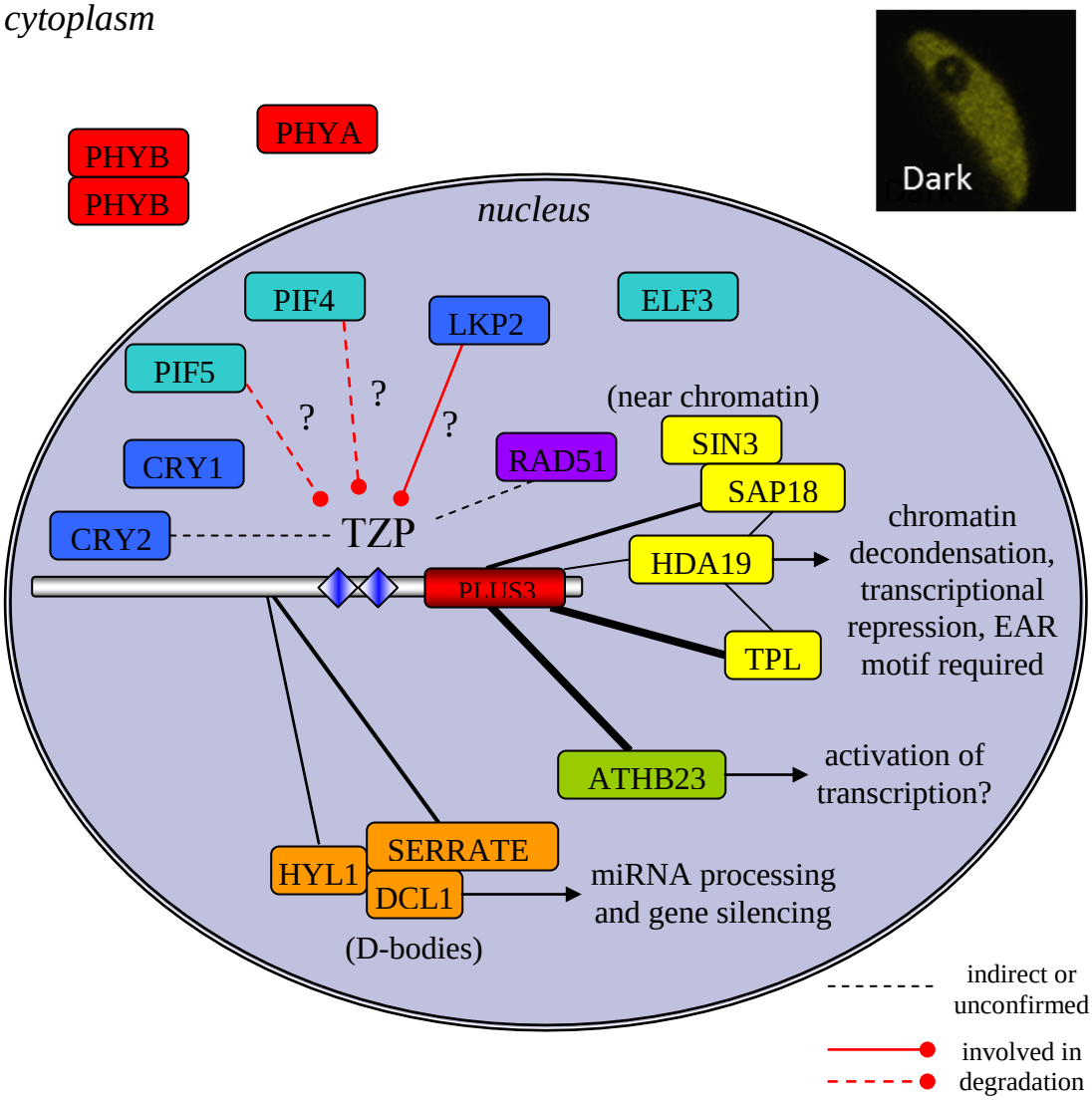
Conclusion

TZP is a potential mediator between gene expression and light signaling as well as the circadian clock. Its protein levels accumulate in blue light and its stability is dependent at least in part on the presence of some members of the blue light signaling pathway. It interacts in yeast with known EAR-motif associated corepressors, like TPL and SAP18, as well as with proteins involved in miRNA processing and LKP2, a member of the Zeitlupe family (refer to schemes 1-3 on the next three pages).

Some more work has to be done to determine if TZP interacts directly with PIF4 and/or PIF5 as well as with members of the Zeitlupe family *in planta*, possibly through the use of BiFC. All of these yeast interactions must also be confirmed *in planta*. The transcript levels of TZP have to be investigated by qPCR in the blue light signaling mutants that show abnormal TZP levels in the dark and/or in blue light. qPCR analysis of other genes like *ATHB2* and *HFR1*, which are among the most up-regulated genes when TZP is overexpressed, in these mutant backgrounds would also reveal whether misregulation of TZP at the protein level in these mutants is correlated with changes in gene expression. NERD was also an interesting candidate for yeast two-hybrid interactions with TZP, but initial attempts to isolate full-length cDNA failed.

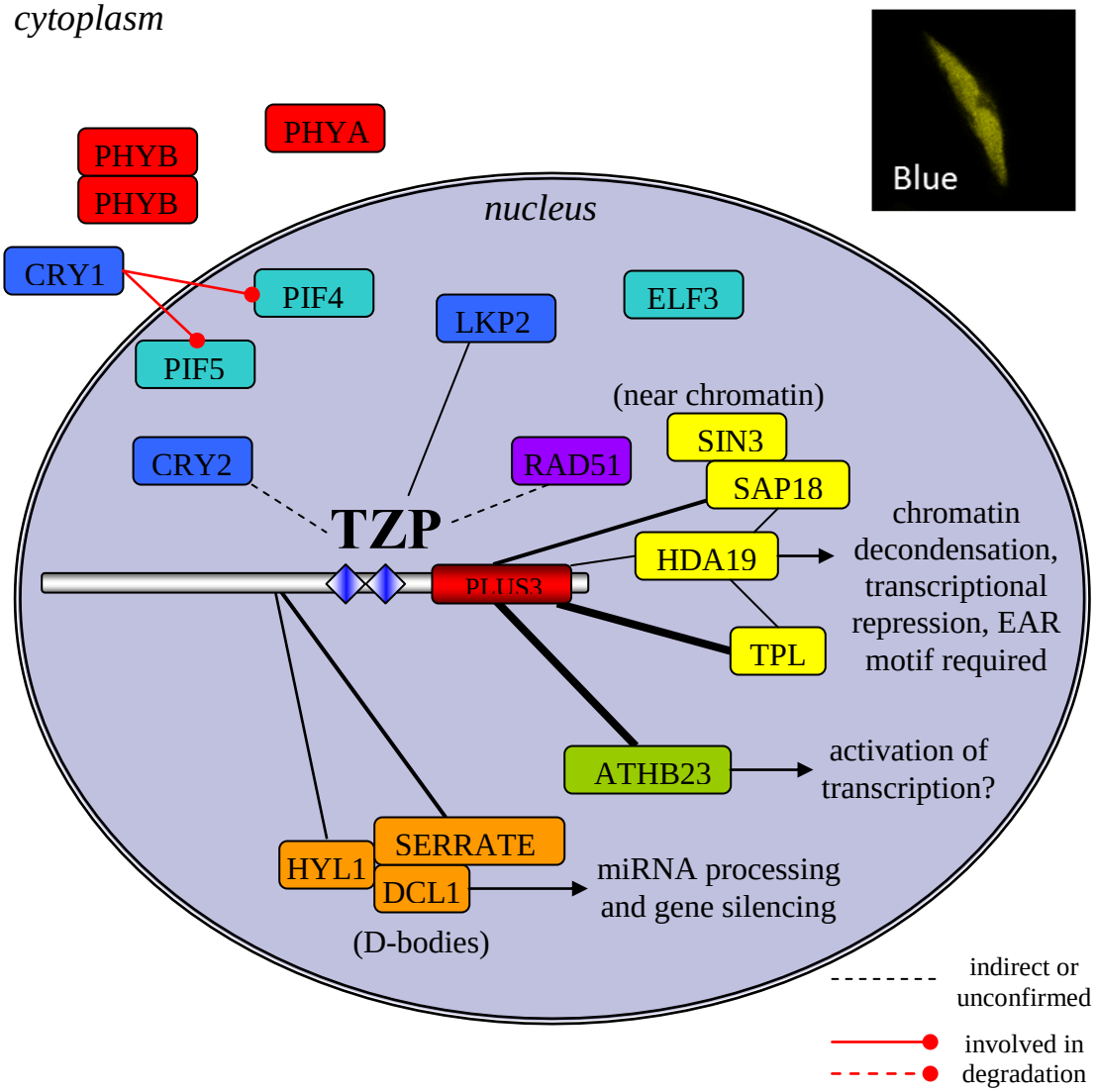
Scheme 1 – Model of TZP Interactions in the Dark

TZP diffusely localized to nucleus, PHYB (inactive P_R) in cytoplasm
lower TZP levels in dark, normally targeted for degradation



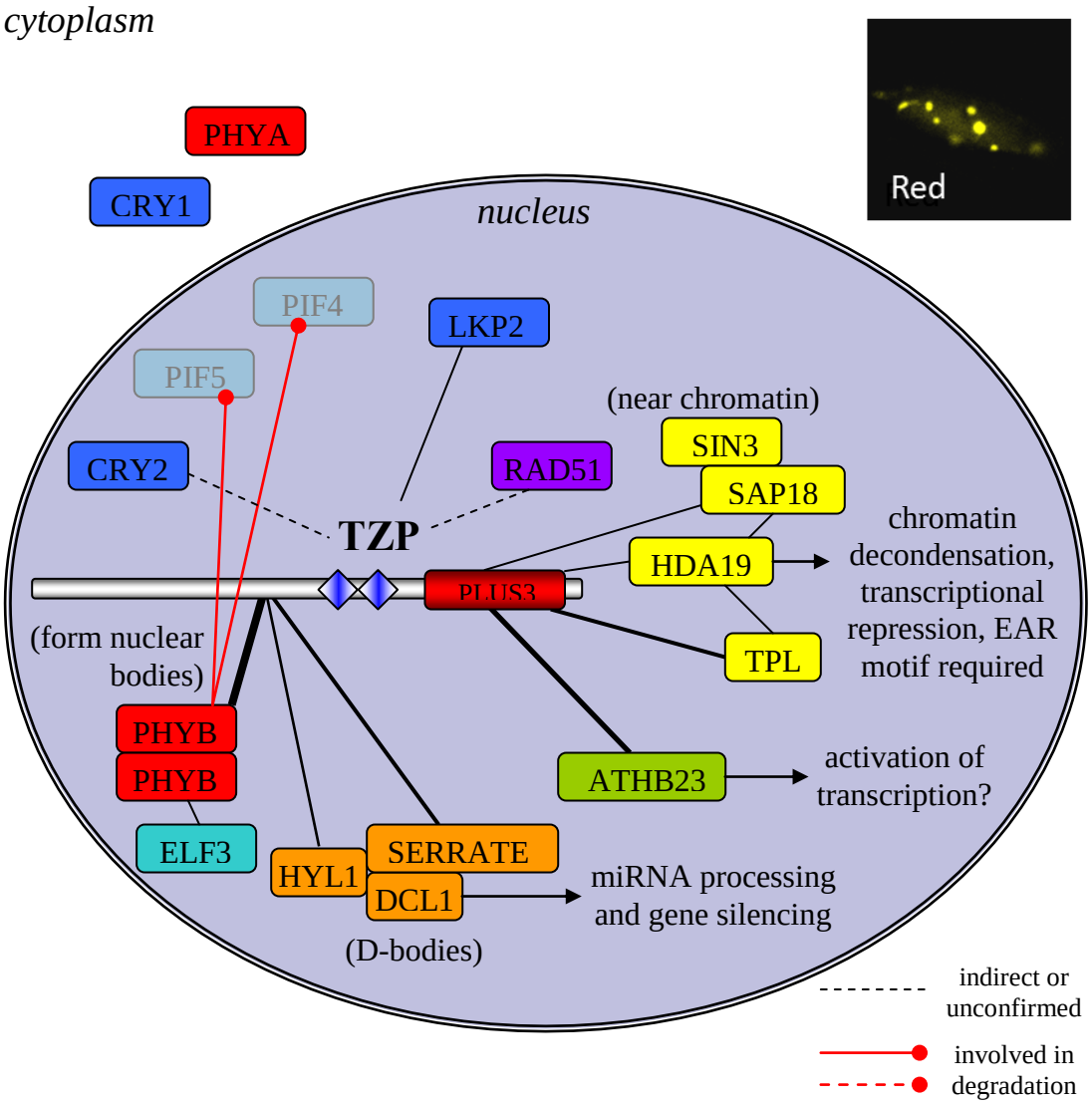
Scheme 2 – Model of TZP Interactions in Blue Light

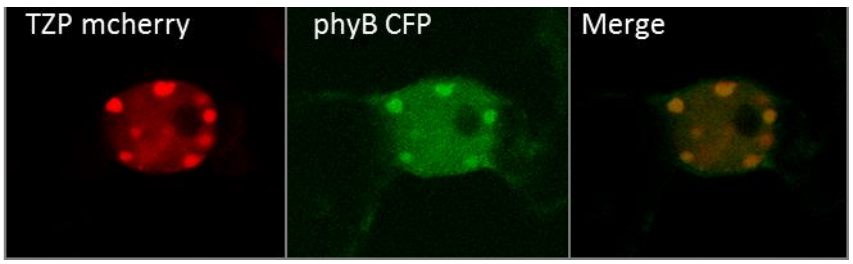
TZP diffusely localized to nucleus, PHYB (inactive P_R) in cytoplasm
TZP accumulates in blue light, protected from degradation



Scheme 3 – Model of TZP Interactions in Red Light

PHYB (active P_{FR}) enters nucleus, interacts with TZP in nuclear bodies





Methods and Materials

Plant growth and light conditions

Arabidopsis thaliana seeds were sterilized using 50% bleach for 3 minutes, then washed three times with sterile MilliQ water. The seeds were then plated on Whattman filter paper atop 1/2 Linsmaier and Skoog (LS) agar (0.8%) plates.

For RNA and protein experiments (except MG132 treatment – see below), seeds were then stratified by placing the plates at 4ºC in the dark for 3 days, followed by 2-hour exposure to light for inducing and synchronizing germination. The plates with seeds were then kept in the dark for up to 4 days. For time course experiments, the plates were placed into the appropriate LED light chamber (Percival Scientific) for the indicated period of time, subtracting backwards from a set time point so that all samples could be collected at approximately the same time. All the light chambers were calibrated using an LI-COR LI-250A light meter so that the seedlings would be exposed to 1 $\mu mol \bullet m^{-2} \bullet s^{-1}$ (µE) of light.

For MG132 treatment, seeds were plated, stratified and place in the dark as described above, but during treatment the seedlings were transferred to MilliQ water with or without MG132 (50 µM in H_2O using 10 mM stock dissolved in DMSO) for a total treatment time of five hours, with at least 1 hour of soaking prior to exposure to light. The seedlings were then transferred into light chamber with continuous exposure to MG132 or water (as a control) for the indicated period of time and all samples were collected at the same time.

Protein extraction and western blots

Seedlings grown in the indicated conditions were flash-frozen in liquid nitrogen. The samples were ground to a fine powder using a mixer mill (Retsch MM300) and metal beads. The protein was then extracted, following the addition of sample buffer (4x stock – 250 mM Tris, 40% glycerol, 20% beta-mercaptoethanol. 2% SDS and 0.5% bromophenol blue), by boiling for 3-5 minutes. After spinning down, the supernatants were loaded onto 4-12% Bis-Tris gels and run using MOPS running buffer. The gels were run until the 25 kDa band of the Bio-Rad Precision Plus Protein™ Dual Color Standard ran off. The protein bands were then transferred onto 0.45 µm nitrocellulose membranes (Bio-Rad) using a standard wet transfer for 1 hour at 100V.

The membranes were blocked with 4% non-fat dry milk in TBS-Triton, followed by primary antibody treatment – 1:5000 dilution of anti-GFP (Roche), 1:1000 dilution of anti-TZP, 1:1000 dilution of anti-DsRed (Clontech) or 1:5000 dilution of anti-UGPase (Agrisera) as a loading control – for 2 days, then washed three times with TBS-Triton prior to secondary antibody treatment – 1:5000 dilution of goat anti-mouse HRP (Bio-Rad) or goat anti-rabbit HRP (Bio-Rad) – for 1-2 hours. All antibodies were applied diluted in 4% milk TBS-Triton. The blots were then washed five times with TBS-Triton prior to exposure using Thermo Scientific SuperSignal® West Pico (for loading control) or Dura. For all experiments, except for truncation lines, each blot was cut at about 75 kDa so that the top could be blotted with anti-GFP, anti-TZP or anti-DsRed, while the bottom could be blotted with anti-UGPase as a loading control. Since some of the truncations were of similar size to UGPase, the blots could not be cut and so they were first blotted with anti-GFP, then stripped with Bioland Scientific LLC Multi-Western™

stripping buffer according to the manufacturer's instructions and reblotted with anti-UGPase.

Yeast Two Hybrid

Competent Mav203 yeast cells were transformed with two of the following constructs to check for interactions – ATHB23-pDEST22, SE-pDEST22, SAP18-pDEST22, SIN3-pDEST22, HDA19-pDEST22, HYL1-pDEST22, LKP2-pDEST22, TZP-pDEST32, TZPRm-pDEST32 (lacking arginines in EAR motif), TZPearMut-pDEST32 (lacking leucines in EAR motif), PLUS3-pDEST32 or one of the empty pDEST vectors for negative controls. All constructs except TZPRm-pDEST32 and TZPearMut-pDEST32, were amplified from plant-derived cDNA using primers that contain flanking attB sequences for insertion into pDONR221 with a BP reaction and then into pDEST22 or pDEST32 with an LR reaction (Clontech). Sequencing using plasmid specific primers (refer to Supplementary Table 1 for sequences) confirmed the presence of the correct cDNA sequence.

The TZPRm mutant was generated using the QuikChange Site-Directed Mutagenesis kit, then inserted into pDEST32 as above. The TZP EAR mutant (LRLRL →ARARA) was created using a two-step PCR reaction with Phusion polymerase. The first step used a plasmid that contains TZP as the template and TZPearMut-forward and attB-reverse or TZPearMut-reverse and attB-forward primers (refer to Supplementary Table 1 for sequences) to create complementary strands that both contained the intended mutation. In the second step, the PCR products from the first step were used as templates in order to amplify the TZP EAR mutant sequence with the attB forward and reverse primers. The resulting PCR product was inserted into pDEST32 as above.

The transformed yeast were then plated onto -Leu/-Trp plates made with 6.7 g/L of Difco™ Yeast Nitrogen Base without amino acids (BD), 20 g/L of glucose, 20 g/L of Bacto™ agar (BD) and -Leu/-Trp or -Leu/-Trp/-His amino acid supplement, with the pH adjusted to 5.6-5.7. After confirming the presence of both constructs, four dilutions of each plasmid combination were spotted onto -Leu/-Trp or onto -Leu/-Trp/-His plates with 0, 10, 25, 50 and 100 mM 3AT concentrations.

The expression of the expected fusion proteins was verified by running yeast protein extracts on 4-12% Bis-Tris gels with MOPS running buffer, then transferred onto nitrocellulose membranes and blotted with primary antibodies (Clontech) against the GAL4 activation domain for pDEST22 (1:5000 dilution) or the DNA-binding domain for pDEST32 (1:10000 dilution) as above. Yeast protein extracts were obtained by resuspending one spot per transformed line in 200 µL of MilliQ water, which were then spun down and the supernatant was replaced with 200 µL of 0.2 mM NaOH (modified from (Kushnirov 2000)). After spinning down, the supernatant was replaced with 30 µL of sample buffer and boiled for 3-5 minutes. After spinning down, the supernatants were loaded onto the gels.

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