A cross-kingdom Nudix enzyme that pre-empts damage in thiamin metabolism

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Synopsis:

Genes specifying the thiamin monophosphate phosphatase and adenylated thiazole diphosphatase steps in fungal and plant thiamin biosynthesis remain unknown, as do genes for thiamin diphosphate (ThDP) hydrolysis in thiamin metabolism. A distinctive Nudix domain fused to thiamin diphosphokinase (Tnr3) in *Schizosaccharomyces pombe* was evaluated as a candidate for these functions. Comparative genomic analysis predicted a role in thiamin metabolism, not biosynthesis, because free-standing homologues of this Nudix domain occur not only in fungi and plants, but also in proteobacteria (whose thiamin biosynthesis pathway has no adenylated thiazole or thiamin monophosphate hydrolysis steps) and animals (which do not make thiamin). Supporting this prediction, recombinant Tnr3 and its *Saccharomyces cerevisiae*, *Arabidopsis*, and maize Nudix homologues lacked thiamin monophosphate phosphatase activity but were active against ThDP, and up to 60-fold more active against diphosphates of the toxic thiamin degradation products oxy- and oxothiamin. Deleting the *S. cerevisiae* Nudix gene (*YJR142W*) lowered oxythiamin resistance, overexpressing it raised resistance, and expressing its plant or bacterial counterparts restored resistance to the *YJR142W* deletant. By converting the diphosphates of damaged forms of thiamin to monophosphates, the Tnr3 Nudix domain and its homologues can pre-empt the misincorporation of damaged diphosphates into ThDP-dependent enzymes, and the resulting toxicity.

Short title: A Nudix enzyme that pre-empts damage in thiamin metabolism

Key words: comparative genomics, metabolite proofreading, Nudix hydrolase, thiamin, vitamin B_1

Abbreviations used: ADT, adenosine diphospho-5-(β-ethyl)-4-methylthiazole-2-carboxylic acid; oxoThDP, oxothiamin diphosphate; oxyThDP, oxythiamin diphosphate; TDPK, thiamin diphosphokinase; ThDP, thiamin diphosphate; ThMP, thiamin monophosphate; ThTP, thiamin triphosphate; TPK, thiamin phosphate kinase; ZmNUDIX, *Zea mays* gene GRMZM2G031461

INTRODUCTION

Thiamin, in its active form thiamin diphosphate (ThDP), is a universal cofactor for transketolases, decarboxylases, and other enzymes that make or break C–C bonds. Most bacteria, fungi, and plants can synthesise thiamin *de novo*, but animals cannot and must obtain it from the diet. Essentially all organisms can convert thiamin to ThDP.

The thiamin synthesis pathway has several variants (Figure 1) [1,2]. In the pathway in fungi, plants, and a small minority of bacteria, the thiazole moiety is made from NAD, glycine, and cysteine by the Thi4 protein, whose adenylated thiazole product (adenosine diphospho-5-(β-ethyl)-4-methylthiazole-2-carboxylic acid, ADT) has an ADP moiety whose diphosphate bond is subsequently cleaved to release AMP [3]. The diphosphatase responsible is not known either as an enzyme or a gene [4].

A second missing gene in the fungal- and plant-type pathway is a phosphatase that hydrolyzes thiamin monophosphate (ThMP) to thiamin, which is then converted by thiamin diphosphokinase (TDPK) to ThDP [2,4]. This phosphatase step is absent from the great majority of bacteria, which use a thiamin phosphate kinase (TPK) to convert ThMP to ThDP directly (Figure 1).

Plant and fungal genes are also missing for steps in thiamin metabolism, including the dephosphorylation of ThDP and the hydrolysis of thiamin triphosphate (ThTP), a little understood thiamin derivative [4]. For plants, an acid phosphatase catalyzing the two-step dephosphorylation of ThDP to ThMP and thiamin was characterised from maize (*Zea mays*) but not cloned [5]. Similarly, various nucleoside diphosphatases from onion (*Allium cepa*) cleaved ThDP to ThMP *in vitro* but were not identified [6]. The physiological significance of the maize and onion enzymes is not known.

There are thus several missing genes for thiamin synthesis or metabolism enzymes that cleave C-O-P or P-O-P bonds. It is consequently interesting that *Schizosaccharomyces pombe* TDPK (Tnr3) has a Nudix family protein fused to its N-terminus [7]. As such fusions ('Rosetta stones') strongly imply a functional association between the fusion partners [8], and as Nudix proteins typically cleave P-O-P bonds and, rarely, C-O-P bonds [9], the Tnr3 Nudix domain is a prima facie candidate for one of the missing diphosphatase or phosphatase genes.

We therefore made a comparative genomics analysis of the Tnr3 Nudix domain and its homologues in other organisms. This analysis predicted a function in metabolism rather than synthesis. The prediction was validated by demonstrating the capacity of these proteins to hydrolyze ThDP and its damage products *in vitro* and *in vivo*. The ability to hydrolyze corrupt forms of ThDP gives Tnr3 and its homologues a pre-emptive role [10] in combating the metabolic damage that such forms can cause.

EXPERIMENTAL

Bioinformatics

Protein sequences were taken from GenBank, MaizeSeqence.org, and the SEED database [11]. Comparative genomics analyses of bacterial genomes were made using SEED database tools [11]. Sequence alignments were made with Multalin [12] or ClustalW [13], and phylogenetic trees were constructed by the neighbor-joining method using MEGA5 [14].

Chemicals and reagents

All biochemicals were from Sigma-Aldrich, except 8-oxo-dGTP, which was from TriLink BioTechnologies. Oxothiamin was a gift from Dr. T.P. Begley (Texas A&M University). BIOMOL GreenTM was from Enzo Life Sciences. Bio-Scale Mini Profinity IMAC cartridges and Bio-Scale Mini Macro-Prep High Q cartridges were from Bio-Rad.

Preparation of oxothiamin and oxythiamin diphosphates

Oxothiamin diphosphate (oxoThDP) and oxythiamin diphosphate (oxyThDP) were synthesised using mouse TDPK as described previously [15] with the following modifications. Reactions (100 µl)

contained 50 mM Tris-HCl, pH 8.0, 15 mM $MgCl_2$, 10 mM ATP, 10 mM oxothiamin or oxythiamin, and 52–130 µg TDPK. After 2 h at 37°C, a further 52–132 µg TDPK were added and incubation was continued for 2 h. A 2-µl aliquot was diluted 100 times in water, protein was removed using a Corning® Spin-X® UF Concentrator (10 kDa), and 100 µl of the flow-through was analyzed by HPLC. Oxythiamin and its phosphate esters were separated isocratically on a Vydac Protein & Peptide C18 column (4.6 mm × 25 cm, 5 µm) in 100 mM triethylammonium acetate, pH 7.0, and detected at 260 nm. Oxothiamin and its phosphate esters were separated on a Beckman Ultrasphere C18 column (4.6 mm × 25 cm, 5 µm) equilibrated with 10 mM potassium phosphate, pH 7.18 (A) and held for 5 min after injection. Over 4 min the mobile phase was changed to 90% A, 10% water, and then to 50% A, 12.5% water, 37.5% methanol over 9 min. Over the next 2 min the mobile phase was changed to 10% A, 15% water, 75% methanol, which was then held for 6 min. The column was recycled to A over the following 4 min and then re-equilibrated in this buffer for 8 min before the next injection. Detection was at 245 nm. OxyThDP and oxoThDP yields were 82% and 100%, respectively. The products of the reaction were directly used for enzymatic assays after removal of TDPK protein as described [15]. As oxyThDP yield was only 82% the amount of deproteinised reaction mixture added to assays was adjusted accordingly.

Constructs for expression in *Escherichia coli*

DNA sequences were amplified using Phusion High-Fidelity DNA polymerase (New England BioLabs). The primers used are given in Supplementary Table S1. All constructs were sequence-verified. *S. pombe* cDNA clone spa102c24 encoding Tnr3 was obtained from the Yeast Genetic Resource Center (Osaka, Japan). The coding region was amplified using primers Tnr3-F and Tnr3-R and cloned between the NdeI and XhoI sites of pET-43.1a, which adds a C-terminal hexahistidine tag. *Saccharomyces cerevisiae* cDNA clone ScCD00100772 encoding Yjr142w was obtained from PlasmID (Harvard Medical School). The coding region was amplified using primers Yjr142w-F and Yjr142w-R and cloned, as for the *S. pombe* cDNA, between the NdeI and XhoI sites of pET-43.1a. These constructs were introduced into *E. coli* Rosetta 2 cells (Novagen) for protein expression. Maize EST clone ZM_BFb0221H02.r encoding GRMZM2G031461 (ZmNUDIX) was obtained from the Arizona Genomics Institute. The open reading frame (minus residues 1–37) was amplified using primers Zmays-F and Zmays-R. The amplicon was digested with AseI and PstI and cloned between the NdeI and PstI sites of pCold II (TaKaRa); this added a hexahistidine tag to the N-terminus. The pCold II expression construct for *Arabidopsis thaliana* At5g19460 (AtNUDT20) minus residues 1–49 was obtained from Dr. S. Shigeoka (Kinki University, Japan) and was as described [16]. The ZmNUDIX and AtNUDT20 constructs were introduced into T7 Express I^q competent *E. coli* cells (New England BioLabs). A pET28b plasmid containing the coding sequence of mTPK (provided by Dr. T.P. Begley) was introduced into Rosetta 2 cells.

Constructs for expression in *S. cerevisiae*

The primers used are given in Supplementary Table S1. The Yjr142w and ZmNUDIX coding sequences were subcloned into pYES2 (Invitrogen). The Yjr142w sequence was amplified from the ScCD00100772 cDNA using primers pYESYNudix-F and pYESYNudix-R. The ZmNUDIX sequence (minus the first 37 residues) was amplified from the ZM_BFb0221H02.r cDNA using primers ZmBF-pYES2-F and ZmBFpYES2-R. Both sequences were cloned between the BamHI and NotI sites of pYES2. Genomic DNA of *Polynucleobacter necessarius* subsp. *asymbioticus* QLW-P1DMWA-1 was a gift from Dr. M.W. Hahn (University of Innsbruck, Austria). Amplification of the Nudix coding sequence was performed on *P. necessarius* genomic DNA or *Arabidopsis* leaf cDNA using primers pY-PolyNud-F and pY-PolyNud-Kpn-R, and pY-AtNUD-Kpn-F and pY-AtNUD-Kpn-R, respectively. The amplicons were digested with KpnI and cloned in KpnI-digested, dephosphorylated pYES2.

Production and purification of recombinant proteins

For protein production, cells were grown at 37°C in LB medium containing 100 µg/ml ampicillin until A_{600} reached 0.5–0.7 (Tnr3 and AtNUDT20) or 0.8–1.0 (Yjr142w and ZmNUDIX). Isopropyl-D-

thiogalactopyranoside (IPTG) was then added (final concentration 400 μM); for Yjr142w and ZmNUDIX, cells were held on ice for 30 min before adding IPTG. Incubation was then continued for 18-24 h at 25°C (Tnr3 and AtNUDT20) or 15°C (Yjr142w and ZmNUDIX). Subsequent operations were at 0–4°C. Cells were harvested by centrifugation (7,500 ***g***, 10 min) and resuspended in 50 mM Tris-HCl pH 8.0, 1 mM β-mercaptoethanol (5 ml per g cell paste). Cells were transferred to 2-ml screw cap tubes two-thirds full of 0.1 mm glass or zirconia/silica beads, and broken in a Mini-Bead-Beater (BioSpec Products). After centrifugation (20,000 ***g***, 30 min), proteins were purified from the supernatant using a BioLogic LP system (Bio-Rad). For Tnr3, Yjr142w, ZmNUDIX, and mTPK, the supernatant was loaded onto a 1-ml IMAC column pre-equilibrated with buffer A (50 mM Tris-HCl pH 8.0, 1 mM β-mercaptoethanol, 0.5 M NaCl), at 0.5 ml/min. After washing with buffer A containing 10 mM imidazole, proteins were eluted using a 20-ml linear gradient of buffer A containing 10–200 mM imidazole at 2 ml/min. Eluted fractions were analyzed by SDS-PAGE, fractions clean of contaminants were pooled, desalted on PD-10 columns (GE Healthcare), concentrated on Microcon YM-10 units (Millipore), and immediately used for enzymatic assays. For AtNUDT20, the supernatant was applied to a 5-ml Macro-Prep High Q column pre-equilibrated with Buffer B (50 mM Tris-HCl pH 8.0, 1 mM β-mercaptoethanol) at 5 ml/min. After washing with 5 volumes of buffer B, proteins were eluted using a 25-ml linear gradient of buffer B to buffer A at 2.5 ml/min. Fractions containing AtNUDT20 were identified by SDS-PAGE, pooled, and NaCl concentration was adjusted to 0.5 M. The sample was then further purified using a 1-ml IMAC column as described above. Proteins were estimated by dye binding [17] with bovine serum albumin as the standard.

Enzyme assays

Assays were routinely made in triplicate in 50-μl reaction mixtures at 37°C for 10–120 min in 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$ containing the specified concentrations of substrates. Reactions were stopped by adding 50 mM EDTA. Hydrolase activities were assayed by detecting inorganic phosphate using the BIOMOL Green™ reagent after adding either 0.5 unit of inorganic pyrophosphatase (Sigma) or 4 units of alkaline phosphatase (Sigma) as described [18]. For determination of activities towards oxoThDP, oxyThDP, and thiamin triphosphate, 10-μl to 40-μl aliquots were adjusted to 200 μl with water, deproteinised by filtration with Corning® Spin-X® UF Concentrators (10 kDa), and 100-μl of the flow-through was analyzed by HPLC as described above. Thiamin and its phosphate esters were analyzed as described for oxythiamin. Kinetic data were analyzed in GraphPad (GraphPad Software) by direct fitting to the Michaelis-Menten equation using nonlinear regression.

Yeast growth assays

Cells of *S. cerevisiae* strain BY4741 or the haploid *YJR142W* deletant (obtained from Euroscarf and verified by genomic PCR), plus or minus the pYES2 vector alone or containing *YJR142W* or plant, animal, or bacterial Nudix genes, were grown at 30°C on yeast nitrogen base medium without amino acids, ammonium sulphate, or thiamin (from FORMEDIUM), and with the following supplements: ammonium sulphate (5 g/l), arginine, cysteine, leucine, lysine, threonine, tryptophan, adenine (all 100 mg/l), aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, valine (all 50 mg/l), (minimal medium), and the specified concentrations of sugars, uracil, and oxythiamin.

Determination of thiamin and its phosphates

S. cerevisiae strain BY4741 cells harboring pYES2 vector alone or containing *YJR142W* were grown for two days at 30°C as above except that 2% raffinose was the sole carbon source, then inoculated (OD_{600} 0.1) in 100 ml of liquid medium as above containing 1% raffinose and 2% galactose. At 9 h (when OD_{600} was 0.3-0.35) oxythiamin was added (final concentration 10 μM). Cells were harvested at 27 h, washed with phosphate buffered saline, frozen in liquid N_2 and stored at -80°C. Cell pellets were resuspended in 2 ml 7.2% perchloric acid and sonicated. The sonicate was held on ice for 15 min with periodic vortex mixing, then cleared by centrifugation at 4°C (2000 ***g***, 15 min). Thiamin and its phosphates were analyzed by oxidation to thiochrome derivatives followed by HPLC with fluorometric detection [19]. The oxidation reagent was a freshly prepared solution of 12.14 mM pot-

assium ferricyanide in 3.35 M NaOH. Samples or standards (160 µl) were mixed with 15 µl methanol; 100 µl of oxidation agent was added, mixed for 60 s, and 100 µl of 1.43 M phosphoric acid was then added. The standards (thiamin, ThMP, ThDP) were made up in 7.2% perchloric acid/0.25 M NaOH (1:1, v/v). Samples (50 µl) were separated on an Alltima HP C18 amide column (150 × 4.6 mm; 5 µm; 190Å; Alltech). The mobile phase (1 ml/min) consisted of a gradient of potassium phosphate (140 mM, pH 7.00)/12% methanol (buffer A) to 70% methanol (buffer B). Runs began with 100% buffer A; within 10 min, the ratio A/B reached 50/50, becoming 0/100 in the following 5 min.

RESULTS

The Tnr3 Nudix domain is highly characteristic and has homologues in other organisms

BlastP searches of the NCBI and SEED databases revealed an orthologue of the *S. pombe* Tnr3 Nudix-TDPK fusion protein in the related fission yeast *Schizosaccharomyces japonicus*, and free-standing homologues of the Tnr3 Nudix domain in *Saccharomyces cerevisiae* (Yjr142w), in other fungi, in some but not all animals, in plants, and in a minority of bacteria (~8% of the 3,400 bacterial genomes in GenBank) (Figure 2A). The overall sequence identity between these proteins and Tnr3 is typically ≥30%. *Arabidopsis* has two such homologues, AtNUDT20 and AtNUDT24, which share 76% identity and are encoded by adjacent genes (At5g19460 and At5g19470, respectively). Maize appears to have only one homologue, GRMZM2G031461 (ZmNUDIX). The *Arabidopsis* and maize proteins, and their counterparts from other plants, have predicted plastid or mitochondrial targeting peptides [16], and proteomics analyses have detected both *Arabidopsis* proteins in plastids [4].

Tnr3 Nudix homologues form a characteristic subfamily within the Nudix family, the homologue(s) in any organism being phylogenetically closer to Tnr3 subfamily members from other organisms than to any Nudix protein from that organism. Thus, among the 26 canonical Nudix proteins in *Arabidopsis* [20, 21], AtNUDT20 and AtNUDT24 form a distinct clade along with Tnr3 Nudix and its *S. japonicus* and bacterial counterparts (Figure 2B). Reciprocally, among the Nudix proteins of *S. pombe*, the plant homologues group with Tnr3 (Supplementary Figure S1A). Tnr3 and its plant homologues likewise group with the Tnr3 homologues of bacteria (Supplementary Figures S1B and S1C). These phylogenetic patterns indicate that Tnr3 Nudix homologues are orthologous to each other.

Sequence alignments (Supplementary Figure S2) show that Tnr3 Nudix orthologues have several distinguishing features. First, the canonical Nudix motif $Gx_5Ex_7REUxEExGU$ (where U is a hydrophobic residue) is nearly always incomplete, particularly in the eukaryotic proteins, where various residues replace the first glutamate and lysine replaces arginine. Second, there are conserved regions of around 60 residues upstream and 50 residues downstream of the Nudix signature region, for each of which a Prosite motif can be defined (Supplementary Figure S2). These Prosite motifs can be used to detect additional members of the subfamily.

Certain bacterial Tnr3 Nudix homologues are thiamin-associated

Comparative genomics analysis using the SEED database and its tools [11] showed that, among the relatively few bacteria with Tnr3 Nudix orthologues, those in the β-proteobacterial genera *Polynucleobacter* and *Neisseria* cluster on the chromosome with thiamin synthesis genes (Figure 2C). In *Polynucleobacter* species the Nudix gene is next to an operonic structure comprising *thiC* and four other *thi* genes, with a fifth close by in opposite orientation, whereas in *Neisseria* species the Nudix gene is next to *thiC* alone (Figure 2C). Like the fusion with the TDPK gene in fission yeasts, such conserved gene clustering connotes a functional association with thiamin [22,23].

Comparative genomic analysis predicts a function in thiamin metabolism, not biosynthesis

Assuming that Tnr3 Nudix orthologues are isofunctional, their occurrence in many animals (Figure 2A) argues strongly against their being either of the missing thiamin biosynthesis enzymes, i.e. ADT diphosphatase or ThMP phosphatase, because animals do not have a *de novo* thiamin biosynthesis pathway. Further evidence against ADT diphosphatase or ThMP phosphatase roles comes from

genome-based reconstruction of the thiamin synthesis pathway in bacteria with Tnr3 Nudix orthologues, e.g. *Polynucleobacter* and *Neisseria* species (Figure 2A). Based on the thiamin-related enzymes encoded by their genomes, these bacteria produce the thiazole moiety of thiamin by the usual bacterial route (Figure 1) and lack Thi4, the fungal- and plant-type enzyme that forms ADT diphosphate; they consequently do not need an ADT diphosphatase. Similarly, these bacteria have the usual bacterial direct conversion route from ThMP to ThDP via TPK (Figure 1) and so do not need a ThMP phosphatase.

While inconsistent with a biosynthetic role, the distribution of Tnr3 Nudix orthologues is fully consistent with a role in thiamin metabolism. Moreover, this role can be predicted to involve ThDP in some way because ThDP occurs in all organisms (made either from thiamin by TDPK or from ThMP by TPK), and because the diphosphate bond in ThDP makes it a likely substrate for a Nudix enzyme. These negative (not biosynthesis) and positive (probably ThDP metabolism) predictions were judged robust enough to warrant investigation of the substrate specificities of Tnr3, *S. cerevisiae* Yjr142w, maize ZmNUDIX, and one of the two similar *Arabidopsis* enzymes (AtNUDT20).

Tnr3 Nudix proteins lack ThMP phosphatase activity but hydrolyse typical Nudix substrates

We first tested the prediction that the Tnr3 protein, Yjr142w, AtNUDT20, and ZmNUDIX are not ThMP phosphatases using purified recombinant proteins (Supplemental Figure S3). The plant proteins were engineered to remove their predicted targeting sequences. Activity was assayed at pH 8.0 in the presence of 5 mM Mg^{2+}. Under these conditions, none of the proteins had detectable activity towards ThMP or other monophosphates (Table 1). These results fit with the negative bioinformatic prediction. No tests were made of ADT diphosphatase activity because ADT diphosphate cannot be prepared in substrate amounts, Thi4 being a single-turnover enzyme [24].

All four proteins showed activity towards certain typical Nudix substrates (nucleoside di- and triphosphates, deoxynucleoside triphosphates, the oxidised nucleotide 8-oxo-dGTP) but not others (sugar nucleotides, NADH, NAD^+, FAD, diadenosine tetraphosphate, and coenzyme A) (Table 1).

Tnr3 Nudix proteins hydrolyze ThDP and ThDP analogues

Unlike their inactivity with ThMP, Tnr3, Yjr142w and AtNUDT20 released orthophosphate from ThDP, and also ThTP, although the activities were far below those with nucleotides (Table 1). Since Nudix enzymes can have higher ('housecleaning') activity with damaged substrates than with native ones [9], we tested the diphosphates of oxothiamin and oxythiamin. Oxothiamin is an oxidation product of thiamin [15] and oxythiamin is a hydrolysis product [25] (Figure 1). Both oxoThDP and oxyThDP were hydrolysed, yielding orthophosphate, far more rapidly (six- to 60-fold) than ThDP by Tnr3, Yjr142w and AtNUDT20, and were also hydrolysed by ZmNUDIX. OxoThDP and oxyThDP were among the best substrates for Tnr3 and Yjr142w.

HPLC analysis of the initial reaction products of representative enzymes (Yjr142w and AtNUDT20) showed that these enzymes initially hydrolyse oxyThDP or oxoThDP to products with the chromatic behaviour expected for the monophosphate forms, not to free oxy- or oxothiamin (Figure 3), i.e. that these enzymes preferentially cleave the diphosphate (P-O-P) bond, releasing orthophosphate. In this, as well as in a general preference for diphosphate over triphosphate substrates, these enzymes resemble the distinctive *E. coli* Nudix enzyme YmfB [26]. A further similarity is that YmfB can, like Yjr142w and AtNUDT20, hydrolyse ThDP and ThDP analogues to their monophosphates [27].

To further investigate substrate preference, kinetic analyses were made with Tnr3 and its *S. cerevisiae* and plant orthologues using ThDP, oxyThDP, and oxoThDP, plus GDP as a benchmark (Table 2). The K_m values for ThDP and its oxy- and oxo-derivatives were similar to each other (within a factor of three) and generally comparable to that for GDP. However, k_{cat} values for oxyThDP and oxoThDP were one to two orders of magnitude higher than for ThDP. Thus, by the criterion of k_{cat}/K_m ratio, all enzymes displayed a marked preference (ranging from three- to 100-fold) for oxyThDP or oxoThDP over ThDP. Although k_{cat} values of the fungal enzymes for oxyThDP and oxoThDP were in the same range as for GDP, those for the plant enzymes were far lower. However, the latter were

truncated to remove predicted targeting peptides and carried an N-terminal hexahistidine tag, whereas the former were not truncated and had a C-terminal tag. The recombinant plant enzymes thus had unnatural N-termini, and this could have affected their activity.

As Nudix activities and specificities are divalent cation-dependent [28], we tested the effect of replacing Mg^{2+} with Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, or Ca^{2+} (0.5 mM) on the activities of Tnr3 and AtNUDT20 towards ThMP, ThDP, and GDP (Table 3). As with Mg^{2+} as cofactor, none of the metal ions led to activity with ThMP, and while Mn^{2+} at 0.5 mM increased the low activities with ThDP it was ineffective at 5 μM, a more physiological value [29]. Of the other metals, only Co^{2+} gave activity, and only with Tnr3. These results indicate that Mg^{2+} is the physiological cofactor for Tnr3 and its orthologues, and confirm that these enzymes have no activity against ThMP and only weak activity against ThDP.

S. cerevisiae Yjr142w confers resistance to oxythiamin

The thiamin degradation product oxythiamin (Figure 1) is toxic because TDPK phosphorylates it to oxyThDP, and oxyThDP can inhibit ThDP-dependent enzymes [30]. The strong preference of the Tnr3 Nudix domain and its homologues for oxyThDP over ThDP could thus in principle allow them to selectively hydrolyse oxyThDP and so confer resistance to this antivitamin *in vivo*. To test this idea, we compared the oxythiamin resistance of wild type and *YJR142W* deletant strains; the deletant had substantially lower resistance (Figure 4A). As a further test, Yjr142w was overexpressed from the pYES2 plasmid in wild type *S. cerevisiae*. Cells overexpressing Yjr142w became markedly more oxythiamin resistant, growing at concentrations as high as 10 μM (Figure 4B).

To confirm that oxythiamin resistance conferred by overexpressing Yjr142w involved protection of thiamin metabolism, we examined intracellular contents of thiamin and its phosphates. Cells harboring vector alone or overexpressing Yjr142w were grown in liquid medium to which oxythiamin was added after 9 h. As expected, growth continued normally in the overexpressing cells but slowed markedly in the vector-alone controls (Figure 5A). At the end of the culture period, thiamin and its phosphates were determined after conversion to their fluorescent thiochrome derivatives (Figure 5B); oxythiamin and its phosphates cannot form thiochromes as they lack the required amino group on the pyrimidine ring (Figure 1). The Yjr142w-overexpressing cells maintained a far higher level of ThDP and a higher ThDP/thiamin ratio than the control cells (Figure 5C). That free thiamin did not accumulate in control cells (its phosphorylation being blocked by competition with excess oxythiamin) may have been due to its efflux to the medium [31].

Plant and bacterial Nudix orthologues also confer resistance to oxythiamin

To test whether homologues of Yjr142w from other organisms also confer oxythiamin resistance, AtNUDT20, ZmNUDIX, and the orthologous Nudix genes from *Polynucleobacter* and zebrafish (Figure 2) were cloned into pYES2 and tested for their effect on the oxythiamin resistance of the *YJR142W* deletant. The *Arabidopsis* AtNUDT20 and *Polynucleobacter* genes increased resistance about as effectively as the native *YJR142W* gene (Figure 6). The maize ZmNUDIX and zebrafish genes had little or no effect (not shown) but, as protein levels were not measured, poor expression of these genes in *S. cerevisiae* cannot be ruled out; moreover, recombinant ZmNUDIX had much lower activity than AtNUDT20 when expressed in *E. coli* (Table 2).

In silico expression analysis suggests a role of plant Nudix proteins in response to stresses

The Genevestigator microarray dataset and tools [32] were used to study expression of AtNUDT20 and AtNUDT24 (Supplemental Figure S4). The data represent both genes together because the microarray did not distinguish them. Expression was markedly induced by salt stress, drought, or drought plus light stress. Induction by drought plus light stress was further increased in the alternative oxidase 1a (AOX1a) mutant, in which superoxide radical (O_2^-) levels are elevated [33]. These data suggest a role for AtNUDT20/AtNUDT24 in adaptation to stress, and are in accord with reported increases in expression of thiamin synthesis genes and accumulation of thiamin and its phosphates in response to abiotic stresses [5,34]. In addition, gene expression was elevated in seedlings

overexpressing the Zat12 zinc-finger protein. As Zat12 has a central role in reactive oxygen and abiotic stress signaling [35], this suggests that AtNUDT20/AtNUDT24 may belong to a Zat12 regulon. Also consistent with regulation by abiotic stress, the stress responsive cis-elements TATATAA [36] and CTAGAAC [37] are present in the AtNUDT20 and AtNUDT24 promoters, respectively [38].

DISCUSSION

Our comparative genomic, biochemical, and genetic results indicate that the Nudix domain of Tnr3 and its free-standing orthologues in fungi, plants, and bacteria can correct an error by a metabolic enzyme, a process known as metabolite proofreading [10,39], and thereby pre-empt damage that would otherwise ensue. The culprit in this case is TDPK, which can mistakenly pyrophosphorylate degraded forms of thiamin such as oxy- and oxothiamin as well as other thiamin analogs [30]. The resulting diphosphates can replace ThDP in the active sites of ThDP-dependent enzymes, thereby inhibiting their activity [30]. Because Tnr3-like Nudix enzymes prefer ThDP analogues to ThDP itself, they can selectively dephosphorylate the analogues and thus prevent their inhibitory effects.

Tnr3 and its Nudix homologues probably also rectify two problems related to that caused by TDPK's imperfect specificity. First, the thiamin moiety of ThDP is chemically and enzymatically labile [15, 25], so that ThDP can give rise directly to damaged diphosphates such as oxoThDP and oxyThDP. Such damaged diphosphates have the same potential repercussions as diphosphates made by TDPK. In this connection, it may be noted that most ThDP-dependent enzymes in plants are in chloroplasts or mitochondria [2], which are thus probably the main sites of ThDP damage. The predicted localization of the plant Tnr3 orthologues thus assigns them to ThDP damage hotspots. The second problem stems from the rather wide specificities of the two kinases that enable salvage of hydroxymethylpyrimidine and hydroxyethylthiazole, and of the later enzymes in thiamin synthesis (Figure 1). These sloppy specificities allow analogues of hydroxymethylpyrimidine [27] and of hydroxyethylthiazole [40] to be phosphorylated and then to enter the synthesis pathway, which converts them to toxic analogues of ThDP. By dephosphorylating such analogues, Nudix enzymes would in effect proofread the end result of a whole series of enzyme errors.

That Tnr3 and its orthologues hydrolyze both oxoThDP and oxyThDP much more rapidly than ThDP is significant because the defects in these two degraded forms are in opposite halves of the thiamin molecule: in the thiazole moiety of oxoThDP and in the pyrimidine moiety of oxyThDP (Figure 1). Thus the enzymes tested are all capable of selectively hydrolysing chemically diverse forms of corrupted ThDP, presumably including others besides those we tested. It is important to note that there are potentially many others, for oxy- and oxothiamin and their diphosphates are convenient model compounds from a large but poorly known range of damaged forms of thiamin. Other damaged forms include desthiothiamin [41] and mixed thiamin disulphides [15], all of which could exist as diphosphates. It is not known which damaged forms of ThDP predominate *in vivo*; whichever these are, they may be better substrates for Tnr3 and its orthologues than oxy- or oxoThDP.

It is also significant that, by the criterion of k_{cat}/K_m ratios, the *S. cerevisiae* Yjr142w enzyme, which strongly affects oxythiamin resistance *in vivo* (Figure 4), was the least selective *in vitro* for oxyThDP relative to ThDP of any of the enzymes that were characterized (Table 2). The Yjr142w k_{cat}/K_m ratio for oxyThDP was only 2.8-fold higher than that for ThDP, versus 100-fold for Tnr3, 3.7-fold for AtNUDT20, and an indeterminately high value for ZmNUDIX. Thus a modest selectivity *in vitro* suffices for damage pre-emption *in vivo*. Furthermore, it is evident that ZmNUDIX effectively pre-empted damage *in vivo* even though its *in vitro* activity toward oxyThDP was 65-fold less than that toward GDP (Table 2), and the intracellular GDP concentration must far exceed that of oxyThDP. Analogous situations have been noted for other Nudix enzymes [42-44]. One possible explanation is that the fluxes involved in hydrolysing damaged thiamin diphosphates are so small that they can be sustained even by an inefficient enzyme working in the presence of a large excess of better substrates [44]. A further possibility, for the Tnr3 Nudix hydrolase at least, is that its fusion to the TDPK domain privileges access to damaged thiamin diphosphates relative to GDP or other substrates.

Because fungi and plants cannot convert ThMP and its damaged forms to the corresponding diphosphates [2,4], hydrolyzing the diphosphates to monophosphates is an effective way to render them harmless. However, in the longer term it is presumably necessary to hydrolyze the monophosphates and to dispose of the resulting damaged thiamin before it can be re-pyrophosphorylated. It is therefore intriguing that plants have a protein (At5g32470 in *Arabidopsis*) in which a haloacid dehalogenase (HAD) domain is fused to a TenA family protein. HAD proteins are typically phosphatases [45], and TenA proteins in other organisms hydrolyze damaged forms of thiamin [1]. This fusion protein is thus a plausible candidate for the ultimate disposal of damaged thiamin monophosphates.

The damage pre-emption role of Tnr3 and its Nudix orthologues has both narrow and broad parallels with other Nudix family enzymes. The narrow parallel is with *E. coli* YmfB, a Nudix protein that, when overexpressed, confers resistance to thiamin analogs and, *in vitro*, hydrolyses ThDP, hydroxymethylpyrimidine diphosphate and their analogues to the corresponding monophosphates [27]. YmfB belongs to a different Nudix subfamily to Tnr3 and its orthologs, and shares ≤18% overall identity with any of them. It thus appears to represent an independent evolutionary origin of a similar proofreading capability. The broader parallel is with the archetypal 'housecleaning' or 'sanitizing' role of MutT-type Nudix enzymes in hydrolysing non-canonical nucleoside triphosphates, whose incorporation into DNA leads to DNA damage and increased mutagenesis [9,46]. However, these enzymes prevent damage to a macromolecule rather than a cofactor, and release diphosphate from triphosphates rather than phosphate from diphosphates. This parallel is thus conceptual, not mechanistic.

The TDPK-Nudix fusion protein Tnr3 provides what may be the first case of a proofreading enzyme in small molecule metabolism that is fused to the enzyme whose error it corrects. Physical associations between proofreading activities and the enzymes they serve are common in DNA and protein synthesis [39]. It has been argued (i) that these associations are advantageous because they allow synthesis and repair to occur without the macromolecule detaching from the synthesis/repair complex, and (ii) that there would be no such advantage for micromolecule repair because of the speed with which micromolecules diffuse [39]. However, the existence of Tnr3 suggests that the latter argument does not always apply, and it seems reasonable *a priori* that the close proximity enforced by fusing an error-prone enzyme to its proofreader could raise the local concentration of the aberrant substrate and so favor proofreading activity.

Lastly, it should be noted that Tnr3 is only one of hundreds of diverse fusion proteins that contain a Nudix domain of unknown function fused to another protein [47]. Our demonstration of a metabolite proofreading role for a Nudix fusion domain could thus provide a paradigm for predicting functions for many other such domains.

AUTHOR CONTRIBUTION

Aymeric Goyer and Andrew Hanson designed the research, made comparative genomic analyses, and wrote the paper. Aymeric Goyer, Ghulam Hasnain, and Océane Frelin carried out cloning work, Aymeric Goyer characterized enzyme activities, Ghulam Hasnain conducted yeast growth experiments, and Ghulam Hasnain, Maria Ralat, and Jesse Gregory performed thiamin analyses. All authors interpreted experimental results.

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FIGURE LEGENDS

Figure 1 Thiamin synthesis pathways and structures of related compounds

Reactions potentially mediated by a Nudix enzyme are shown with a question mark. Dashed arrows are thiamin degradation reactions leading to oxothiamin or oxythiamin (boxed). ADT, adenosine diphospho-5-(β-ethyl)-4-methylthiazole-2-carboxylate; AIR, aminoimidazole ribotide; DXP, deoxyxylulose 5-phosphate; GAP, glyceraldehyde 3-phosphate; HET, hydroxyethylthiazole; HMP, hydroxymethylpyrimidine; -P, phosphate; -PP, diphosphate; PLP, pyridoxal 5´-phosphate; ThDP, thiamin diphosphate; ThMP, thiamin monophosphate; TDPK, thiamin diphosphokinase; TPK, thiamin phosphate kinase.

Figure 2 Comparative genomic analysis of homologues of the *S. pombe* Tnr3 Nudix domain

(**A**) Distribution among representative eukaryotes and bacteria of genes encoding homologues of the *S. pombe* Tnr3 Nudix domain (Nudix) and of key thiamin synthesis and salvage genes. TDPK, thiamin diphosphokinase; TPK, thiamin phosphate kinase; Thi4, ADT-forming enzyme. Filled boxes denote gene presence; empty boxes denote absence. Bars joining boxes show gene fusions.
(**B**) Phylogenetic tree of the 26 canonical Nudix proteins from *Arabidopsis* plus the *S. pombe* Tnr3 Nudix domain and its *S. japonicus*, *Polynucleobacter* sp. and *Neisseria meningitidis* homologues.
(**C**) The Nudix-TDPK Tnr3 fusion genes in *S. pombe* and *S. japonicus*, and the chromosomal context of the Tnr3 Nudix homologues in *Polynucleobacter* sp. and *N. meningitidis* showing clustering with thiamin biosynthesis (*thi*) genes, colored various shades of green. The white gene with a dotted outline in *Polynucleobacter* sp. is unrelated to thiamin. Arrows indicate the direction of transcription.

Figure 3 HPLC analysis of products formed from oxyThDP or oxoThDP by Tnr3 orthologues

Initial reaction products formed after 10 min incubation were analyzed directly by HPLC. These products had retention times distinct from free oxy- or oxothiamin, and consistent with the presence of a phosphate group. Formation of free oxy- or oxothiamin was not detectable in the reaction conditions used (not shown). Control reactions contained no enzyme.
(**A**) Formation of oxythiamin monophosphate (oxyThMP) from oxyThDP by *S. cerevisiae* Yjr142w. The 50-µl reaction mixture contained 20 nmol of oxyThDP and 4.5 µg of enzyme.
(**B**) Formation of oxothiamin monophosphate (oxoThMP) from oxoThDP by AtNUDT20. The 50-µl reaction mixture contained 20 nmol of oxoThDP and 32 µg of enzyme.

Figure 4 Effect of ablating or overexpressing Yjr142w on *S. cerevisiae* oxythiamin resistance

(**A**) Triplicate isolates of wild type (WT) strain BY4741 and the *YJR142W* deletant (*yjr142w*Δ) were cultured on liquid minimal medium, minus thiamin, plus 0.1 mg/ml uracil and 2% glucose. Serial ten-fold dilutions were then spotted onto plates of the same medium containing various oxythiamin concentrations. Growth was for 36 h at 30°C.
(**B**) Strain BY4741 was transformed with pYES2 vector alone (pYES2) or encoding Yjr142w (Yjr142w). Three independent transformants were cultured on liquid minimal medium, minus uracil and thiamin, plus 2% raffinose. Serial ten-fold dilutions were then spotted onto plates of the same medium with 1% raffinose, 2% galactose, and various concentrations of oxythiamin. Growth was for 6 d at 30°C. No oxythiamin resistance was seen when galactose was omitted (not shown).

Figure 5 Effect of overexpressing Yjr142w on levels of thiamin and its phosphates in *S. cerevisiae*

(**A**) Growth curves of wild type strain BY4741 harboring pYES2 vector alone (pYES2) or encoding Yjr142w (Yjr142w) cultured at 30°C on liquid minimal medium, minus uracil and thiamin, plus 1% raffinose and 2% galactose. Oxythiamin (final concentration 10 µM) was added at 9 h; cells were harvested at 27 h. Data are means ± S.E.M. for three independent transformants; where error bars are absent, they are smaller than the symbol.

(**B**) Representative fluorometric HPLC analyses of thiamin and its phosphates (as their thiochrome derivatives) from equal amounts of cells harboring pYES2 vector alone (pYES2) or encoding Yjr142w (Yjr142w). Note that oxythiamin and its phosphates cannot form thiochromes.
(**C**) Contents of thiamin and its phosphates of cells harboring pYES2 vector alone (pYES2) or encoding Yjr142w (Yjr142w), and the ThDP/thiamin ratio. Data are means and S.E.M. for analyses of three independent transformants. Contents of thiamin and its phosphates are expressed in units of pmol per ml of culture with an OD at 600 nm of 1.

Figure 6 Effect of expressing plant or bacterial Nudix orthologs on oxythiamin resistance of the *S. cerevisiae YJR142W* deletant strain

Triplicate isolates of the *YJR142W* deletant harboring the pYES2 vector alone (pYES2) or encoding Yjr142w (Yjr142w) (as a positive control), AtNUDT20, or the orthologous protein from *Polynucleobacter necessarius* (*Polynucleobacter*) were cultured on liquid minimal medium, minus uracil and thiamin, plus 2% raffinose. Serial ten-fold dilutions were then spotted onto plates of the same medium with 1% raffinose, 2% galactose, and 0, 2, or 3 μM oxythiamin. Growth was for 90 h at 30°C.

Table 1 Substrate preferences of Tnr3 and its *S. cerevisiae* and plant orthologues

Substrate concentrations were 0.4 mM. Activities were determined at 37°C in 50 mM Tris-HCl, pH 8.0, containing 5 mM $MgCl_2$. Results are means ± S.E.M.

Substrate	Specific activity (nmol min^{-1} mg^{-1})			
	Tnr3	Yjr142w	AtNUDT20	ZmNUDIX
ThMP	<0.1	<0.1	<0.1	<0.1
CMP	<0.1	<0.1	<0.1	<0.1
GMP	<0.1	<0.1	<0.1	<0.1
ThDP	9.8 ± 1.0	68 ± 2	2.1 ± 0.2	<0.1
oxyThDP	466 ± 38	284 ± 26	13.0 ± 0.9	1.8 ± 0.2
oxoThDP	657 ± 76	973 ± 100	13.8 ± 0.6	18.1 ± 1.7
ThTP	8.6 ± 0.5	78 ± 4	1.8 ± 0.4	<0.1
CDP	624 ± 5	115 ± 6	103 ± 1.3	58.5 ± 0.5
GDP	525 ± 37	956 ± 27	223 ± 8.6	127.4 ± 1.6
TTP	99 ± 5	43 ± 3	27.6 ± 8.9	9.8 ± 0.5
ATP	124 ± 3	143 ± 3	29.3 ± 7.9	12.6 ± 0.5
GTP	90 ± 11	178 ± 6	33.2 ± 7.0	17.6 ± 0.3
CTP	136 ± 1	196 ± 3	59.2 ± 0.3	21.7 ± 0.7
dGTP	129 ± 4	181 ± 4	4.9 ± 1.1	2.6 ± 0.1
dCTP	204 ± 5	99 ± 3	12.2 ± 5.6	5.0 ± 0.1
dATP	92 ± 5	238 ± 4	7.7 ± 5.2	2.9 ± 0.1
dTTP	99 ± 2	170 ± 2	16.4 ± 6.3	2.1 ± 0.1
8-oxo-dGTP	521 ± 7	809 ± 31	16.3 ± 8.0	5.5 ± 0.7
ADP-ribose	<0.1	<0.1	<0.1	<0.1
UDP-glucose	<0.1	<0.1	<0.1	<0.1
NADH	<0.1	<0.1	<0.1	<0.1
NAD^+	<0.1	<0.1	<0.1	<0.1
FAD	<0.1	<0.1	<0.1	<0.1
Ap_4A	<0.1	<0.1	<0.1	<0.1
Coenzyme A	<0.1	<0.1	<0.1	<0.1

Table 2 Kinetic constants of Tnr3, Yjr142w, AtNUDT20, and ZmNUDIX

Results are means ± S.E.M.

Enzyme	Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Tnr3	GDP	856 ± 350	1.695 ± 0.544	1980
	ThDP	338 ± 137	0.020 ± 0.004	59
	oxyThDP	108 ± 43	0.643 ± 0.074	5950
	oxoThDP	985 ± 308	2.219 ± 0.539	2250
Yjr142w	GDP	467 ± 52	2.030 ± 0.144	4350
	ThDP	305 ± 33	0.085 ± 0.005	276
	oxyThDP	773 ± 506	0.592 ± 0.283	766
	oxoThDP	417 ± 257	1.415 ± 0.488	3390
AtNUDT20	GDP	768 ± 269	0.418 ± 0.104	545
	ThDP	327 ± 49	0.002 ± 0.000	7
	oxyThDP	1270 ± 351	0.033 ± 0.006	26
	oxoThDP	944 ± 207	0.028 ± 0.004	30
ZmNUDIX	GDP	612 ± 81	0.199 ± 0.017	326
	ThDP	-	<0.00006	-
	oxyThDP	680 ± 0	0.003 ± 0.000	5
	oxoThDP	667 ± 346	0.025 ± 0.007	38

Table 3 Divalent metal ion preferences of Tnr3 and AtNUDT20 proteins

Metal ion concentrations were 0.5 mM unless specified otherwise. Substrate concentrations were 0.4 mM. No activity for either enzyme with any substrate was detected with Ni^{2+}, Cu^{2+}, Zn^{2+} or Ca^{2+}. (not shown). Results are means ± S.E.M.

Metal ion	Specific activity (nmol min^{-1} mg^{-1})					
	Tnr3			AtNUDT20		
	ThMP	ThDP	GDP	ThMP	ThDP	GDP
Mg^{2+}	<0.1	1.3 ± 0.1	183 ± 12	<0.1	<0.1	34 ± 0.9
Mn^{2+}	<0.1	1.6 ± 0.1	76 ± 1	<0.1	1.3 ± 0.1	161 ± 0.6
Mn^{2+} (5 μM)	<0.1	<0.1	<0.1	<0.1	<0.1	1.2 ± 0.6
Co^{2+}	<0.1	1.2 ± 0.4	38 ± 7	<0.1	<0.1	<0.1

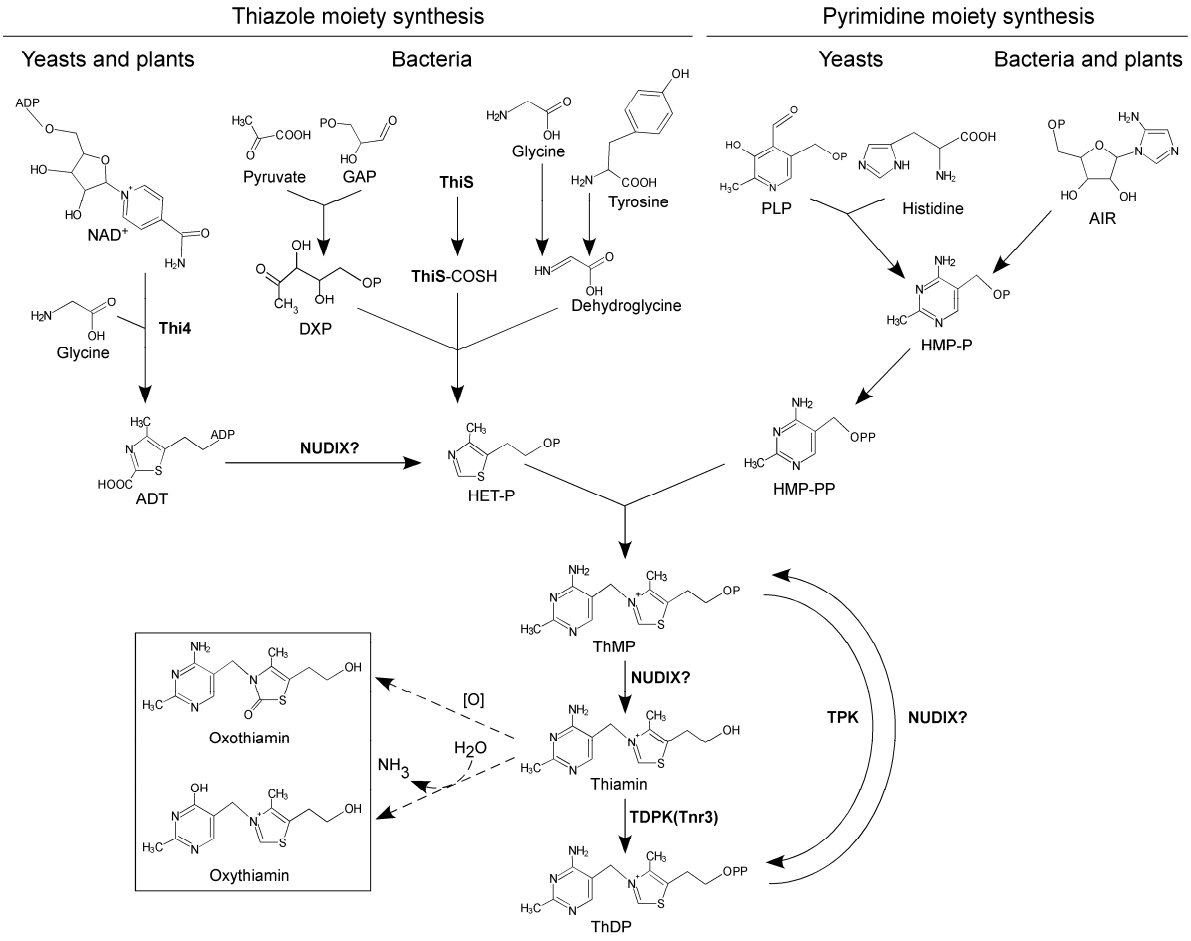
Thiazole moiety synthesis
Pyrimidine moiety synthesis
Yeasts and plants
Bacteria
Yeasts
Bacteria and plants
NAD+
Pyruvate
GAP
ThiS
Glycine
Tyrosine
PLP
Histidine
AIR
Glycine
Thi4
DXP
ThiS-COSH
Dehydroglycine
HMP-P
ADT
NUDIX?
HET-P
HMP-PP
ThMP
NUDIX?
TPK
NUDIX?
[O]
H2O
NH3
Oxothiamin
Thiamin
TDPK(Tnr3)
Oxythiamin
ThDP

Figure 1

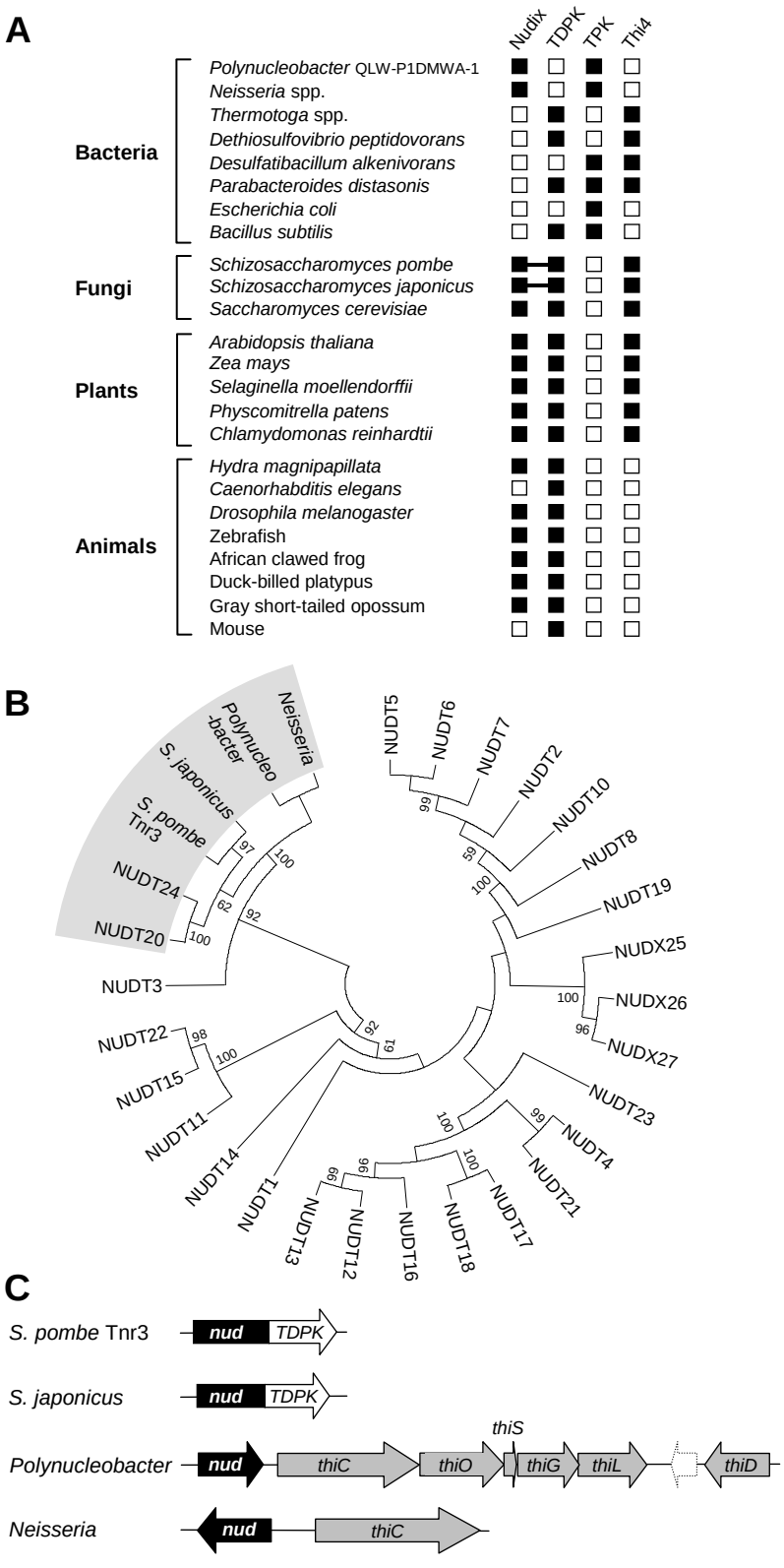
A
Nudix
TDPK
TPK
Thi4
Bacteria
Polynucleobacter QLW-P1DMWA-1
Neisseria spp.
Thermotoga spp.
Dethiosulfovibrio peptidovorans
Desulfatibacillum alkenivorans
Parabacteroides distasonis
Escherichia coli
Bacillus subtilis
Fungi
Schizosaccharomyces pombe
Schizosaccharomyces japonicus
Saccharomyces cerevisiae
Plants
Arabidopsis thaliana
Zea mays
Selaginella moellendorffii
Physcomitrella patens
Chlamydomonas reinhardtii
Animals
Hydra magnipapillata
Caenorhabditis elegans
Drosophila melanogaster
Zebrafish
African clawed frog
Duck-billed platypus
Gray short-tailed opossum
Mouse
B
Neisseria
Polynucleo-bacter
S. japonicus
S. pombe Tnr3
NUDT24
NUDT20
NUDT3
NUDT22
NUDT15
NUDT11
NUDT14
NUDT1
NUDT13
NUDT12
NUDT16
NUDT18
NUDT17
NUDT21
NUDT4
NUDT23
NUDX27
NUDX26
NUDX25
NUDT19
NUDT8
NUDT10
NUDT2
NUDT7
NUDT6
NUDT5
C
S. pombe Tnr3
nud
TDPK
S. japonicus
Polynucleobacter
thiS
thiC
thiO
thiG
thiL
thiD
Neisseria

Figure 2

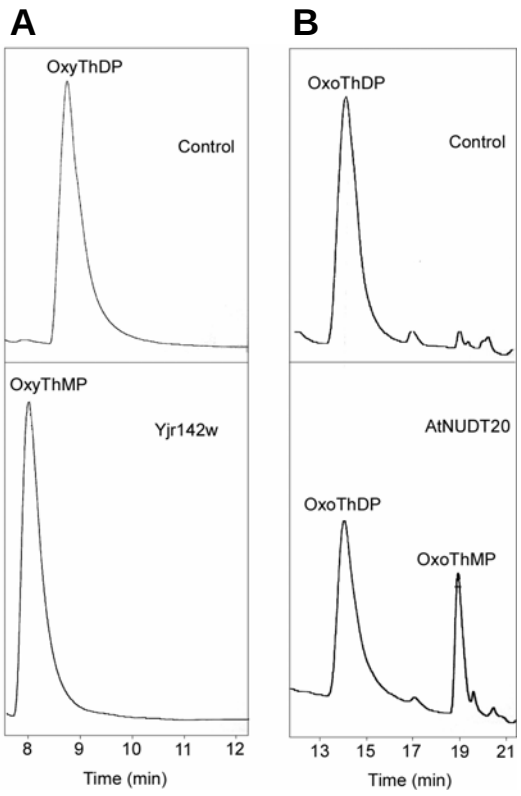
A
B
OxyThDP
Control
OxoThDP
Control
OxyThMP
Yjr142w
AtNUDT20
OxoThDP
OxoThMP
8
9
10
11
12
Time (min)
13
15
17
19
21
Time (min)

Figure 3

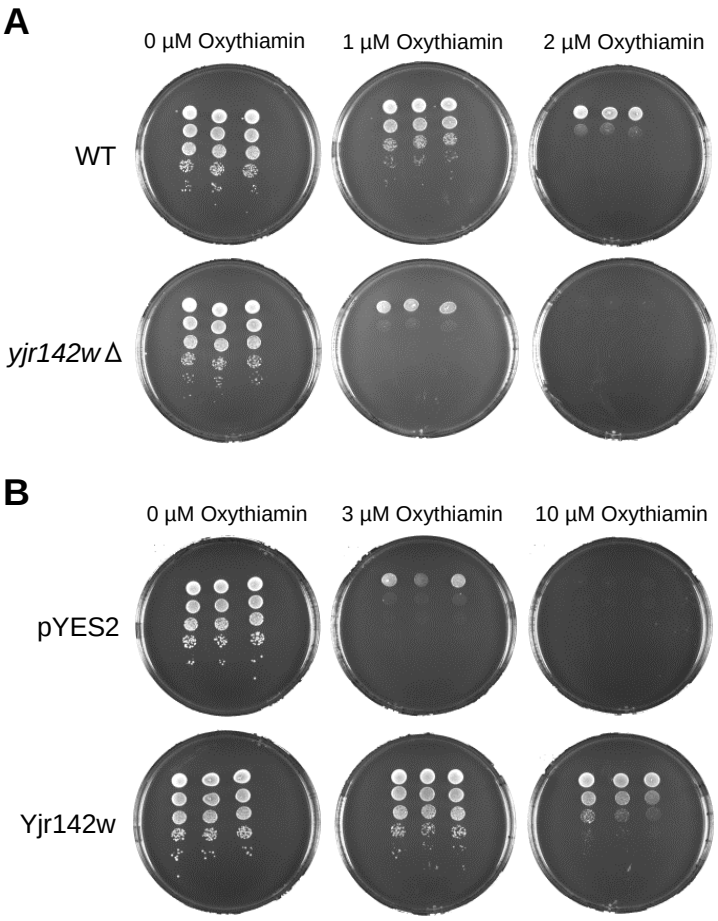
A
0 µM Oxythiamin
1 µM Oxythiamin
2 µM Oxythiamin
WT
yjr142wΔ
B
0 µM Oxythiamin
3 µM Oxythiamin
10 µM Oxythiamin
pYES2
Yjr142w

Figure 4

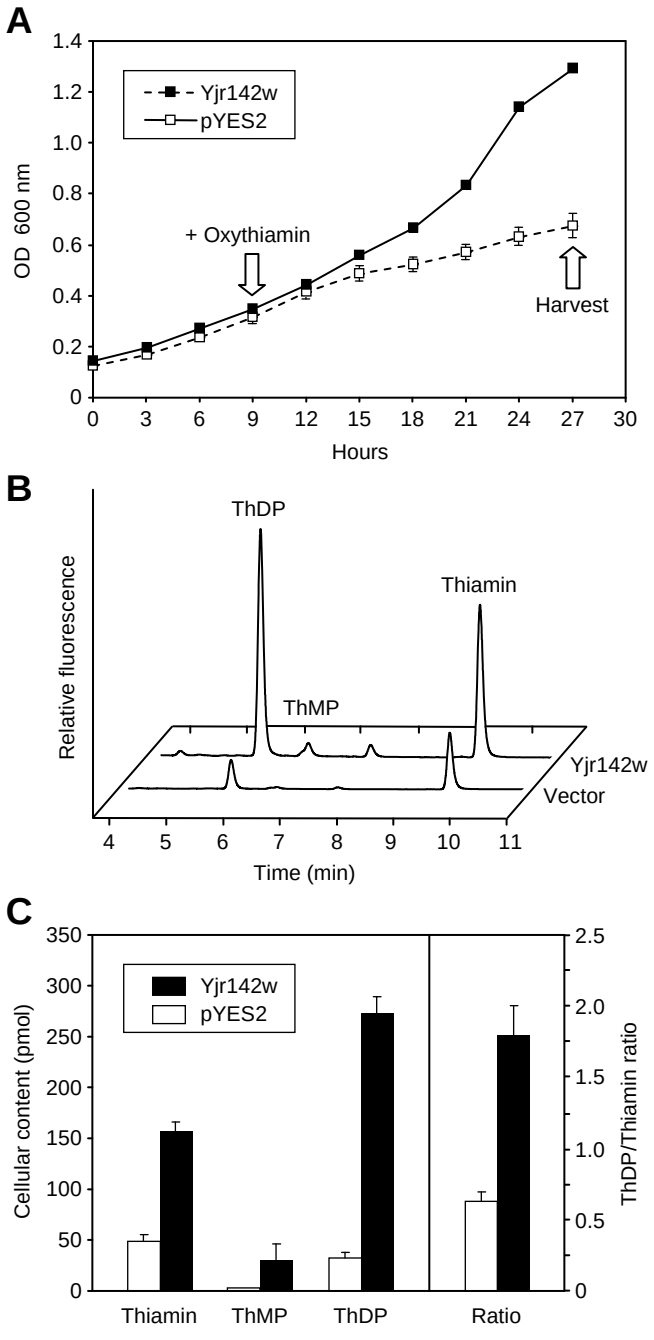
A
OD 600 nm
Hours
Yjr142w
pYES2
+ Oxythiamin
Harvest
B
Relative fluorescence
Time (min)
ThDP
Thiamin
ThMP
Yjr142w
Vector
C
Cellular content (pmol)
ThDP/Thiamin ratio
Yjr142w
pYES2
Thiamin
ThMP
ThDP
Ratio

Figure 5

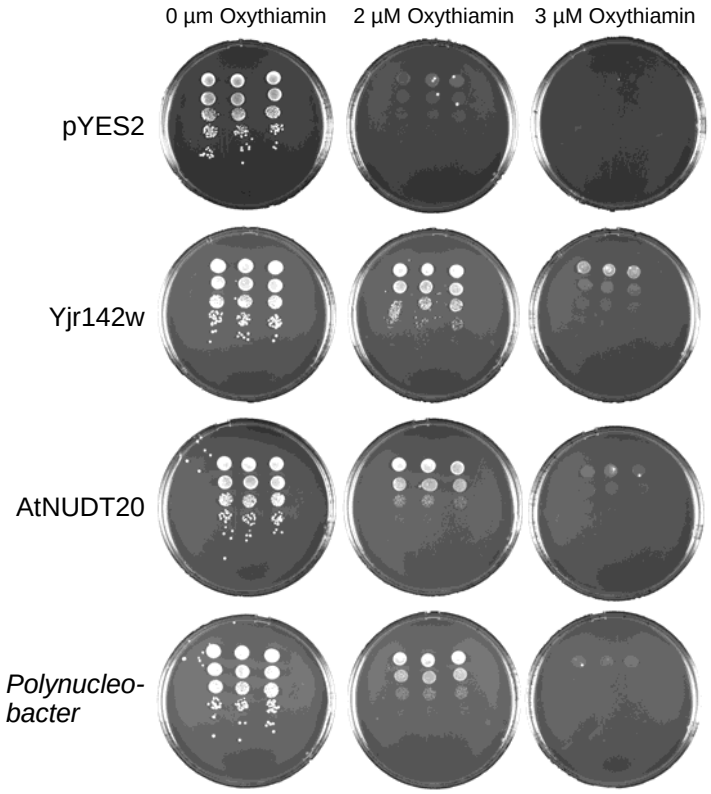
0 µm Oxythiamin
2 µM Oxythiamin
3 µM Oxythiamin
pYES2
Yjr142w
AtNUDT20
Polynucleo-
bacter

Figure 6