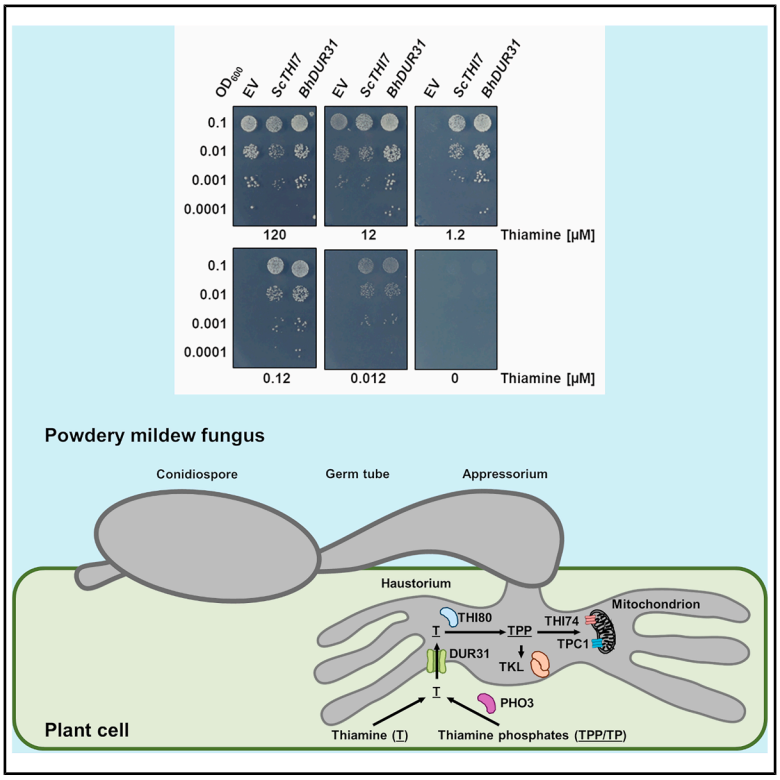
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Article

Thiamine is a vitamin for plant-pathogenic powdery mildew fungi

Graphical abstract



Highlights

- Powdery mildew fungi lost the capacity to synthesize thiamine (vitamin B1)
- *Blumeria hordei* has a functional thiamine transporter and an active THI80 protein
- *B. hordei* transketolase requires thiamine as an essential co-factor for its activity
- Powdery mildew fungi seem to take up thiamine from their plant hosts

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In brief

Plant biochemistry; Interaction of plants with organisms; Molecular plant pathology; Plant pathology

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Thiamine is a vitamin for plant-pathogenic powdery mildew fungi

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SUMMARY

Thiamine (vitamin B1) is an essential micronutrient in all forms of life that serves as a cofactor for several enzymes in primary metabolism. Plant-pathogenic and obligate biotrophic powdery mildew fungi appear to be auxotrophic for the micronutrient, as they have lost the majority of the genes needed for thiamine biosynthesis. Using the barley powdery mildew pathogen, *Blumeria hordei*, as a study object, we found that (1) asexual conidiospores contain detectable levels of thiamine, (2) the *B. hordei THI80* gene encodes a functional thiamine pyrophosphokinase, and (3) *B. hordei* has a functional DUR31-like thiamine transporter likely localizing to the cell surface. We further demonstrated a requirement of the metabolite for the activity of *B. hordei* transketolase, an enzyme of central carbon metabolism known to be thiamine-dependent in other organisms. Taken together, our data suggest that thiamine is a vitamin for *B. hordei* and, thus, likely all powdery mildew fungi.

INTRODUCTION

Powdery mildew fungi are phytopathogens belonging to the Ascomycota. As they pursue an obligate biotrophic lifestyle, they can only grow and reproduce on living plant tissue, which provides essential nutrients required for their survival.[1] Powdery mildew fungi colonize the epidermal cell layer of host plant organs, and mycelial growth occurs mostly on the leaf surface. The asexual pathogen life cycle starts with the germination of a conidiospore. Subsequently, at the end of the germ tube, an appressorium (hyphal "pressing" organ) is formed at the site where the underlying plant cuticle and cell wall are penetrated. If successful, this permits the establishment of a dedicated infection structure, the haustorium, inside epidermal cells. This structure is surrounded by a plant-derived extrahaustorial membrane, which enables the fungus to have close contact with the host cell. The proposed function of the haustorium is the secretion of effector proteins to manipulate the plant cell to its favor and suppress plant immunity but also to take up nutrients from its host.[2,3] Analysis of the genome of the barley powdery mildew pathogen, *Blumeria hordei*, revealed the loss of several biosynthetic pathways, including the one for thiamine (2-[3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5-yl] ethanol), also commonly known as vitamin B1.[4]

Thiamine is a small, water-soluble molecule and the precursor of an essential cofactor for several enzymes in primary metabolism in all forms of life. Accordingly, it is a micronutrient that is indispensable for cellular survival and growth in both pro- and eukaryotes.[5–8] As in the course of the evolution of higher animals, including humans, the capacity for its biosynthesis was lost, thiamine is considered a vitamin in their diet.[9,10] By contrast, bacteria, fungi, and plants are generally deemed to be thiamine autotrophs.[6–8] Inside cells, thiamine is converted enzymatically into its bioactive form, thiamine pyrophosphate (TPP). TPP acts as a cofactor for several metabolic enzymes, including transketolase[11] and pyruvate dehydrogenase,[12] where it serves as a prosthetic group.[13]

Arguably, the best-studied model organism for the biosynthesis and uptake of thiamine is the baker's yeast *Saccharomyces cerevisiae*, which can either synthesize thiamine *de novo* or absorb it from its extracellular environment by membrane-localized transporters (Figure 1). The secreted acid phosphatase Pho3p[14] converts extracellular TPP to thiamine, which is then taken up via the yeast's main plasma-membrane-localized thiamine transporter, Thi7p.[15,16] Besides Thi7p, two additional plasma membrane transporters, Thi71p and Thi72p, also display minor thiamine uptake ability.[17] The Thi7 (alternative name Thi10) thiamine transporter is unique to the Saccharomyces, whereas many other fungal genera utilize thiamine transporter proteins that are similar to DUR31 of *Candida albicans*.[18,19] Inside the yeast cell, thiamine is re-converted by the thiamine pyrophosphokinase Thi80p to TPP,[20] which either is used as an enzymatic co-factor in the cytosol or is transported into mitochondria via the organelle-localized thiamine transporters Thi74p or Tpc1p.[17,21]

The key enzyme for *de novo* thiamine biosynthesis in yeast is Thi6p,[22] which phosphorylates 4-methyl-5-(2-hydroxyethyl)

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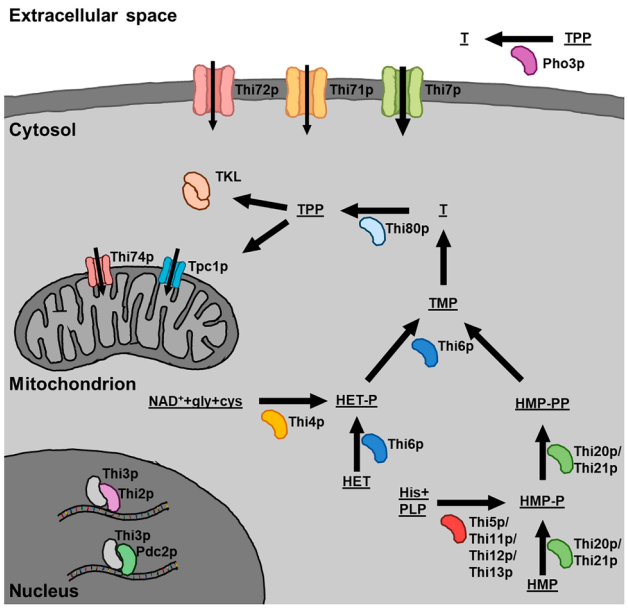


Figure 1. Thiamine biosynthesis and uptake pathways in *S. cerevisiae*

Thiamine (vitamin B1) is an essential cofactor for all living organisms and can be taken up from the environment or synthesized *de novo*. The acid phosphatase Pho3p dephosphorylates thiamine pyrophosphate (TPP) in the extracellular space. Thiamine (T) is absorbed from the extracellular environment by the main thiamine transporter Thi7p (Thi10p) and phosphorylated by Thi80p to its bioactive form thiamine pyrophosphate (TPP). Thi71p and Thi72p can also transport thiamine but play only a minor role. TPP is used as a cofactor by enzymes that have a role in primary metabolism, such as transketolase. Most of these enzymes are located in mitochondria. Tpc1p and Thi74p transport TPP into the mitochondria. The biosynthetic pathways can take different routes to produce different precursors. Thi6p can synthesize the precursor 4-methyl-5-(2-hydroxyethyl) thiazole phosphate (HET-P) via 4-methyl-5-(2-hydroxyethyl) thiazole (HET) or Thi4p by means of NAD^+, glycine (Gly), and cysteine (Cys). 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate (HMP-P) can be synthesized by Thi20p or Thi21p via 4-amino-2-methyl-5-hydroxymethylpyrimidine (HMP) or by the enzymes Thi5p, Thi11p, Thi12p, and Thi13p using histidine (His) and pyridoxal-5-phosphate (PLP). HMP-P is further converted to 4-amino-2-methyl-5-hydroxymethylpyrimidine pyrophosphate (HMP-PP). Thi6p uses HMP-PP and HET-P to produce thiamine monophosphate (TMP), which gets converted to thiamine by dephosphorylation. At lower TPP levels, Thi3p associates with transcriptional activators Thi2p and Pdc2, respectively. In the nucleus, these protein dimers bind to specific promoter regions to activate the expression of genes encoding thiamine biosynthesis enzymes. At higher TPP levels, Thi3p binds TPP and dissociates from Thi2p and Pdc2, leading to transcriptional arrest of these genes.

thiazole (HET) to 4-methyl-5-(2-hydroxyethyl) thiazole phosphate (HET-P). Furthermore, it fuses HET-P with 4-amino-2-methyl-5-hydroxymethylpyrimidine pyrophosphate (HMP-PP) with thiamine monophosphate (TMP). TMP gets dephosphorylated to thiamine, which in turn acts as a substrate for the biosynthesis of TPP via Thi80p as described above. The HMP-PP required for the coupling with HET-P by Thi6p is generated by Thi20p[23,24] and Thi21p[23] from 4-amino-2-methyl-5-hydroxymethylpyrimidine (HMP) and 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate (HMP-P). HMP-P can also be generated by Thi5p, Thi11p, Thi12p, and Thi13p[25] from histidine and pyridoxal-5-phosphate (PLP). HET-P can be synthesized by Thi4p[26,27] from nicotinamide adenine dinucleotide (NAD^+), glycine, and cysteine (Figure 1).

Only a few reports exist regarding the uptake and biosynthesis pathway of thiamine in other (plant-pathogenic) fungi. The transporter in the wilt pathogen *Verticillium dahliae*, a homolog of yeast Thi7p, has been identified, and respective knock-out mutant strains are affected regarding growth and pathogenicity.[28] Moreover, two genes of the thiamine biosynthetic pathway (coding for enzymes catalyzing the biosynthesis of HMP-PP and HET-P) were reported to be highly expressed in haustoria of the bean rust fungus *Uromyces fabae*.[29]

Here, we examined the genomes of 16 different powdery mildew species for the presence/absence of genes related to the biosynthesis and uptake of thiamine and measured the thiamine content of *B. hordei* conidiospores. We identified a yeast *Thi80* homolog in the genomes of three powdery mildew fungi (including *B. hordei*) and verified its function by complementation assays in yeast. Furthermore, we identified a *B. hordei* thiamine transporter that can complement a thiamine-auxotrophic yeast quadruple mutant. Finally, we show that the heterologously expressed *B. hordei* transketolase is dependent on TPP in enzymatic assays. Taken together, we identified the major components of the thiamine uptake pathway of the barley powdery mildew pathogen, which might partially explain the obligate biotrophic lifestyle of this fungus.

RESULTS

Powdery mildew fungi lost essential components of the thiamine biosynthetic pathway

We performed TBLASTN searches with the well-characterized thiamine-associated proteins of *S. cerevisiae* (Figure 1) and the thiamine transporter Dur31 of *C. albicans* against 19 publicly accessible genomes of 16 different powdery mildew species. Apart from an assessment regarding the presence/absence of the respective genes in these genomes, we also estimated the respective copy numbers based on the quantity of TBLASTN hits obtained and their assignment to different genomic scaffolds/contigs (Figure 2; Table S1). For presumed single-copy genes (*THI80*, *THI3*, *THI74*, and *DUR31*—see below), we further extracted the protein sequences of annotated powdery mildew genomes from the EnsemblFungi database to judge the sequence conservation of the proteins within the powdery mildew lineage. In some of these genomes, these genes are either not or wrongly annotated, which leads to a variable number of powdery mildew species included in these alignments (Figures S1–S4).

In most of the inspected genomes, we did not detect any of the thiamine-biosynthesis-related genes (BLAST e-value threshold $1e^{-5}$), except for *Golovinomyces magnicellulatus* and *Parauncinula polyspora.* The genomes of the latter two species contain almost all genes examined for, except a *Thi6* homolog, which encodes one of the most important enzymes in this pathway as it catalyzes the synthesis of the immediate thiamine precursor, TMP (Figure 1). Reciprocal TBLASTN searches revealed that the respective hits in the genomes of *Golovinomyces magnicellulatus* and *Parauncinula polyspora* most likely derive from sequences of non-powdery mildew microbes (primarily fungi but

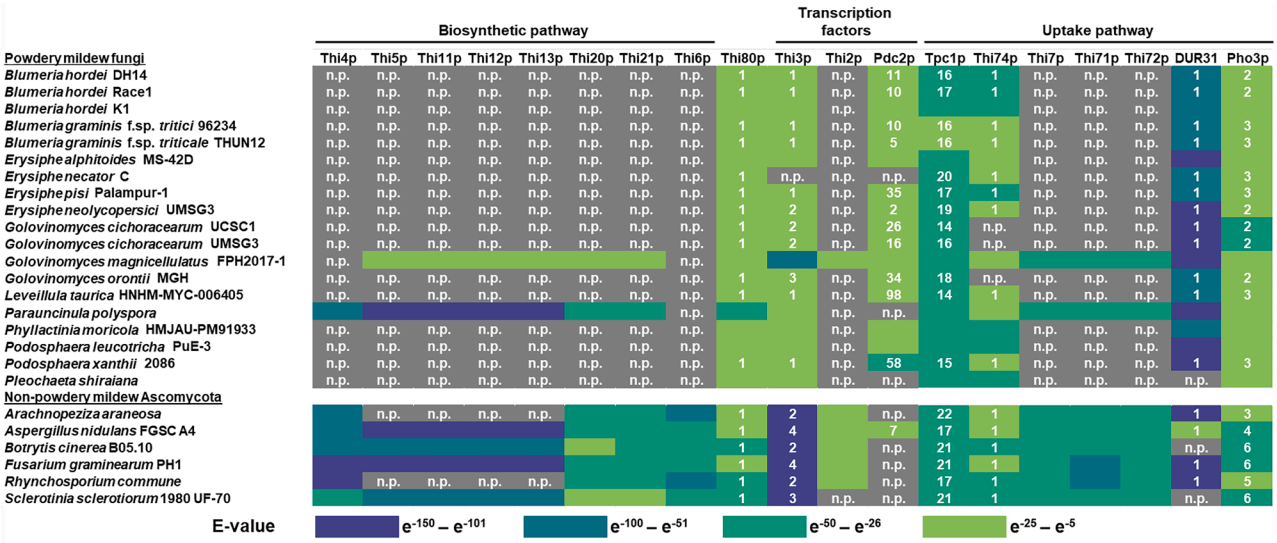


Figure 2. *In silico* analysis of thiamine-related genes in powdery mildew fungi and selected other ascomycetes

TBLASTN searches of well-characterized thiamine proteins of *S. cerevisiae* and the *C. albicans* thiamine transporter Dur31 were performed against 19 genomes of 16 different powdery mildew species and the genomes of six selected non-powdery mildew ascomycetes. The e-values of the BLAST hits are visualized as a heatmap, where dark blue depicts the highest range of e-values (e^{-150} to e^{-101}) and light green the lowest range of e-values (e^{-25} to e^{-5}). All genes with hits below the indicated BLAST threshold (>$1e^{-5}$) are indicated as n.p. (not present). An estimate of the putative copy number (indicated as a number in the respective box) is given for each gene that is present in the majority of the powdery mildew species and for which a genome with a BUSCO score of ≥89% exists.

also bacteria) that contaminate the genome assemblies of these two species (Table S2). Other tested ascomycetes, like the closest known relative of the powdery mildew fungi, *Arachnopeziza araneosa*,[30] or well-known plant pathogens such as *Sclerotinia sclerotiorum*, *Fusarium graminearum*, or *Botrytis cinerea,* harbor homologs of the biosynthetic pathway genes in their genomes. However, *A. araneosa* and *Rhynchosporium commune* lack recognizable homologs to *Thi5*, *Thi11 Thi12,* and *Thi13*, which are required for the histidine/pyridoxal-5-phosphate-based branch of thiamine biosynthesis (Figure 1).

All inspected fungal genomes contain one homolog of *Thi80*, encoding the enzyme that converts thiamine into TPP (Figure 1). The amino acid sequence of THI80 is highly conserved in large parts of the protein. The fungi of the Erysiphe clade have smaller insertions at different positions in the protein, which range from a few amino acids to almost 50 amino acids in the case of the extended N-termini of the *Erysiphe neolycopersici* and *Erysiphe pisi* THI80 variants (Figure S1). In baker's yeast, three key transcription factors, Thi2p,[31] Thi3p,[32] and Pdc2p,[12] regulate the thiamine metabolism by controlling the expression of several *Thi* genes[33] (Figure 1). Genes coding for THI2 are either absent or BLAST hits have a low e-value (around $1e^{-4}$) in the powdery mildew genomes. By contrast, we recognized homologs of genes encoding THI3 (1–3 copies) and PDC2 (multiple putative copies) in most powdery mildew genomes, except for *Erysiphe necator* (lacking both) as well as *P. polyspora* and *Pleochaeta shiraiana* (lacking PDC2). Most powdery mildew fungi have one or two potential copies of *THI3*, except for *Golovinomyces orontii*, which harbors three putative copies. The multiple sequence alignment revealed a high conservation of THI3 within the powdery mildews (Figure S2). The other tested ascomycetes have 2–4 copies of this gene. The situation for *PDC2* seems more complex since the potential copy number ranges from two in *E. neolycopersici* to 98 in *Leveillula taurica.* In the other tested ascomycetes, homologs encoding PDC2 seem to be absent, except for *Aspergillus nidulans*, which has seven putative copies of this gene (Figure 2; Table S1).

We found in the genomes of all tested species at least one homolog coding for the mitochondrial transporter THI74 and several presumed copies for the mitochondrial transporter TPC1 (Figure 2; Table S1). The THI74 multiple sequence alignment again revealed a high conservation of the amino acid sequences between different powdery mildew species (Figure S3). Within the powdery mildew fungi, only the *G. magnicellulatus* and *P. polyspora* genomes have a putative homolog coding for the yeast's main thiamine transporter Thi7p and the secondary transporters Thi71p and Thi72p, which also likely derive from contaminating non-powdery mildew sequences (Table S2). By contrast, the genomes of all examined powdery mildew fungi contain a single highly sequence-related *DUR31* homolog, except for *P. shiraiana*. The other tested ascomycetes harbor homologs of all transporters (TPC1, THI7, THI71, THI72, THI74, and DUR1), except for *S. sclerotiorum* and *B. cinerea*, whose genomes lack a recognizable *DUR31* homolog (Figure 2; Table S1). At the amino acid level, DUR31 is conserved within the tested powdery mildew fungi. Fungi from the Erysiphe clade lack an N-terminal part that is present in *B. hordei* and *Golovinomyces cichoracearum*, which could, however, be the result of faulty genome annotations. *G. cichoracearum* additionally has a 17-amino acid insertion that is absent in the DUR31 variant of the other three species (Figure S4). We further detected 2–3 putative copies per powdery mildew species for the gene encoding

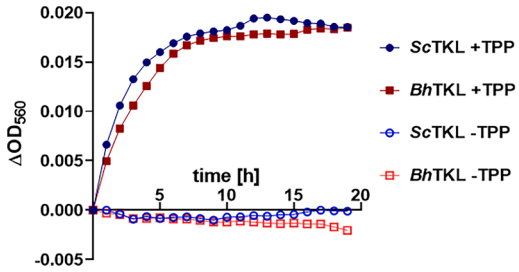


Figure 3. *Bh*TKL requires TPP as a co-factor for enzymatic activity

The enzymatic activity of recombinant *B. hordei* and *S. cerevisiae* transketolase enzymes (*Bh*TKL [blue] and *Sc*TKL [red], respectively) was assessed in a colorimetric assay by measuring the change in absorbance at 560 nm over 20 h following the addition of the purified recombinant proteins to the reaction mixes. Plotted on the y axis is the difference in absorbance to the start of the reaction (ΔOD_{560}). Closed symbols represent reactions performed in the presence of TPP (2.4 mM) and open symbols reactions performed in the absence of TPP. Shown are values averaged from three independent experimental replicates.

the secreted acid phosphatase PHO3, which converts TPP into thiamine in the extracellular space prior to its uptake via dedicated transporters[14] (Figure 1) while the other tested ascomycetes have a slightly higher copy number for this gene (3–6 copies per species; Figure 2; Table S1).

In summary, we conclude that in the course of evolution, powdery mildew fungi lost the majority of the genes coding for enzymes of the thiamine biosynthetic pathway. However, they retained the genes encoding the thiamine/TPP converting enzymes PHO3 (extracellular) and THI80 (cytoplasmic), key nuclear transcription factors (THI3 and PDC2), as well as plasma membrane (DUR31) and mitochondrial (TPC1 and THI74) thiamine transporters. Estimated copy numbers of all these genes are similar to those in the genomes of non-powdery mildew Ascomycota (Figure 2; Table S1), and the amino acid sequences of these proteins within the powdery mildew lineage are largely conserved (Figures S1–S4).

The *B. hordei* genome encodes enzymes that are known to require TPP in other organisms

Given the conservation of part of the thiamine-utilizing machinery in the powdery mildews described above, there are two possible scenarios: these fungi either lost their dependence on TPP/thiamine and neo-functionalized the remaining components in the course of evolution or, alternatively, they are still reliant on vitamin B1 but similar to higher animals and some other microbes became auxotrophic for it. If the latter was true, thiamine might be retrieved from plant host cells, presumably via the fungal haustoria, which supposedly represent key sites of nutrient uptake,[34,35] and could harbor dedicated plasma-membrane-localized transporters.

To approach this question, we first queried whether the *B. hordei* genome codes for proteins that are known to require TPP as a co-factor in other organisms. These are chiefly enzymes involved in primary metabolism ([36]; Table S3). We found that of the 27 enzymes searched for, the *B. hordei* genome encodes seven (Table S3). Previously, a common motif of approximately 30 amino acid residues in length was detected in enzymes that utilize TPP. This sequence stretch starts with the highly conserved sequence glycine-aspartate-glycine (GDG), followed by 20–30 less-defined amino acids, and ends with the highly conserved sequence asparagine-asparagine (NN;[37]). We found one or two copies of the respective motif (or slightly modified versions thereof) in the amino acid sequences of five of the seven *B. hordei* enzymes presumed to be TPP-dependent (Data S1). We additionally analyzed the amino acid sequences of these enzymes by Interpro (https://www.ebi.ac.uk/interpro/), which revealed that all seven enzymes have predicted TPP binding sites, indicated by the presence of domain IPRO29061 (Table S3). In summary, although powdery mildew fungi are known to have lost a marked number of protein-coding genes during evolution,[4,38,39] they retained genes encoding for enzymes that are known to require TPP as a co-factor in other organisms.

B. hordei transketolase requires TPP as a co-factor for enzymatic activity

To test exemplarily the requirement for TPP by these *B. hordei* enzymes, we opted for the heterologous expression of *B. hordei* transketolase (TKL) to perform activity assays with the recombinant protein in the presence or absence of TPP. TKL is an enzyme of the pentose phosphate pathway where it catalyzes the thiamine-diphosphate-dependent transfer of a two-carbon fragment from d-xylulose-5-phosphate to the aldose erythrose-4-phosphate, yielding fructose-6-phosphate and glyceraldehyde-3-phosphate. We chose TKL, as this enzyme unlike many other TPP-dependent enzymes localizes in the cytoplasm, operates as a homodimer, and is not part of an organelle-localized multimeric protein complex.[11,40,41] We cloned and heterologously expressed the transketolases of *B. hordei* (*Bh*TKL, EnsemblFungi: BLGH_05210) and *S. cerevisiae* (Tkl1p, EnsemblFungi: YPR074C; positive control—hereafter referred to as *Sc*TKL) as N-terminally glutathione-*S*-transferase (GST)-tagged proteins in *Escherichia coli*. *Sc*TKL and *Bh*TKL share extensive sequence similarity, and *Bh*TKL harbors conserved amino acids known to be required for substrate and co-factor (TPP and $MgCl_2$) binding in *Sc*TKL[42–44] (Figure S5). Following glutathione affinity purification from crude bacterial lysates and proteolytic removal of the GST tags (Figure S6), the two recombinant transketolase proteins were used in a colorimetric assay[45] to assess their enzymatic activity. In the presence of all required components, *Sc*TKL and *Bh*TKL caused a marked increase in OD_{560} during the first 5 hours of measurement in the colorimetric assay, suggestive of TKL activity. Thereafter, the rise in OD_{560} slowed down and the values plateaued after around 10 hours, yielding typical saturation kinetics (Figure 3). Both enzymes showed comparable activity in this assay as judged from the similar reaction curves. In the absence of TPP, there was almost no change in OD_{560} over the time of measurement, indicating a lack of enzymatic activity in this condition (Figure 3). Additional control reactions lacking either a further known transketolase cofactor ($MgCl_2$) or the reaction educt (lithium hydroxypyruvate) also resulted in a lower ($MgCl_2$) or nearly no (lithium hydroxy-pyruvate) increase in OD_{560} (Figure S7). The latter was also true for empty vector controls (GST only) subjected to this assay (Figure S8). Taken together, the outcome of this

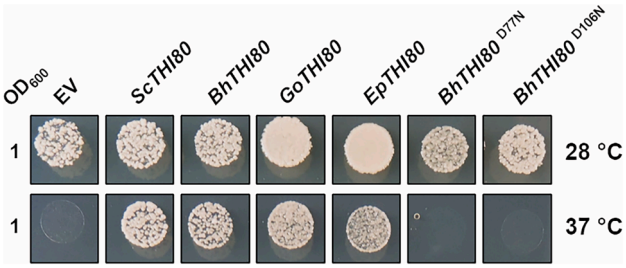


Figure 4. *B. hordei*, *G. orontii*, and *E. pisi* possess a functional thiamine pyrophosphokinase

Cells of a baker's yeast (*S. cerevisiae*) thermosensitive *thi80* mutant strain were transformed with either empty vector (EV, negative control) or expression constructs encoding the native baker's yeast (*S. cerevisiae*) *THI80* (*ScTHI80*, positive control), *B. hordei THI80* (*BhTHI80*), *G. orontii THI80* (*GoTHI80*), and *E. pisi THI80* (*EpTHI80*). For *BhTHI80*, site-directed mutants in key residues of the active site were generated and tested (D77N or D106N). All genes were under the control of the native yeast *THI80* promoter and terminator sequences. Depicted is the outcome of yeast cultures at $OD_{600} = 1$, dropped on plates with SC medium, supplemented with the antibiotic G418. Plates were incubated for 3 days at either 28°C (permissive temperature, upper panel) or 37°C (restrictive temperature, lower panel). The experiment was repeated three times with the same outcome.

experiment suggests that *Bh*TKL is an enzyme that requires TPP as an essential co-factor for its activity.

Conversion of thiamine to TPP by *B. hordei* THI80

Given the presence of TPP-dependent enzymes in the absence of thiamine/TPP biosynthetic machinery in *B. hordei*, we reasoned that the fungal phytopathogen may receive either TPP and/or thiamine from its host plant, barley (*Hordeum vulgare*). In the case of the latter, *B. hordei* should have the competence to convert thiamine to TPP—a biochemical reaction typically catalyzed by thiamine pyrophosphokinase (THI80) proteins.[20] As the powdery mildew genomes retained the genes encoding this enzyme (see above and Figure 2), we speculated that these might be functional. To test this notion experimentally, we took advantage of a thermosensitive *thi80* yeast mutant (YOR143C (Ts747-1).[46] In *S. cerevisiae*, *THI80* is an essential gene, precluding fungal growth in the absence of an active enzyme. The thermosensitive yeast mutant YOR143C (Ts747-1) is viable at the permissive temperature of 28°C but fails to grow at the restrictive temperature of 37°C.[46]

We cloned the *THI80* homologs from *B. hordei*, *G. orontii*, and *E. pisi* (two further powdery mildew species) as well as yeast *THI80* (positive control) under the control of the native yeast *THI80* promoter and terminator sequences and expressed these in the above-mentioned thermosensitive *S. cerevisiae thi80* mutant. All transformants including the empty vector control grew at the permissive temperature, as expected. By contrast, at the restrictive temperature, the empty vector transformant failed to grow, whereas the transgenically expressed THI80 proteins complemented the genetic defect in the thermosensitive *thi80* mutant strain, suggesting the functionality of the three powdery mildew THI80 enzymes (Figure 4). To validate the activity of *Bh*THI80 (EnsemblFungi: BLGH_04864) further, we mutated key active site residues that are known to be essential for pyrophosphate transfer to thiamine in *S. cerevisiae* Thi80p,[47] and conserved in *Bh*THI80 (Figure S9), by site-directed mutagenesis. Transformants of the yeast thermosensitive *thi80* strain expressing these *Bh*THI80 variants (D77N and D106N, corresponding to D97 and D126 in *S. cerevisiae* Thi80p) grew at the permissive temperature but failed to grow at the restrictive temperature, indicating that these mutated enzymes are inactive. In summary, this set of experiments demonstrates that the tested powdery mildew THI80 proteins have the expected thiamine pyrophosphokinase activity. It further suggests that powdery mildews retrieve thiamine, possibly from their host plants, which is then converted to TPP by THI80 in the cytoplasm of these fungi.

B. hordei conidiospores contain detectable levels of thiamine

Given that based on our findings powdery mildew fungi likely obtain thiamine from their plant hosts, we next aimed to detect thiamine in fungal structures. As powdery mildew fungi are obligate biotrophs, they cannot be cultured axenically. During pathogenesis, fungal structures (hyphae and haustoria) are tightly associated with their host plants, preventing reliable experimental dissection at a large scale for metabolite analytics. We, therefore, focused on the asexual powdery mildew propagation units (conidiospores), which can be readily harvested from sporulating colonies on leaf tissue by vacuum aspiration.[4] We collected conidiospores from *B. hordei* colonies and extracted water-soluble metabolites in acidified water as recommended for thiamine.[48] To validate our extraction and analysis protocols, in parallel, we also performed identical extraction from wild-type yeast cells cultured in a rich (thiamine-containing) medium. To detect and quantify thiamine, we used a derivatization method by oxidizing thiamine to the highly fluorescent thiochrome.[49] Subsequent high-performance liquid chromatography-fluorescence detector (HPLC-FLD) analysis revealed a clear peak corresponding in its retention time to the derivatized thiamine standard (Figure 5A). The calculated thiamine accumulation levels in *B. hordei* spores were in the range of 0.4 nmol/mg of fresh weight, whereas thiamine concentrations in yeast samples were almost 50 times higher (Figure 5B). Overall, this analysis confirmed the presence of thiamine in *B. hordei* conidiospores, although at relatively low concentrations.

Apart from the peak corresponding to thiamine, our HPLC-FLD chromatograms obtained during the analysis of yeast and conidiospore extracts contained two additional prominent peaks at the retention times overlapping with the column dead volume (Figure 5A). Such retention times indicate these peaks correspond to relatively polar compounds that do not interact with the reverse-phase column used in our analysis. We hypothesized that these peaks might, at least partially, represent thiamine phosphate esters. To test this hypothesis, we treated crude conidiospore extracts with alkaline phosphatase to release free thiamine from the presumed conjugates, which is one of the methods to analyze total thiamine levels.[49] HPLC-FLD analysis of the phosphatase-treated samples revealed a significant increase in thiamine concentrations in yeast extracts but not in *B. hordei* conidiospore samples (Figure 5B). Overall, this indicated that, unlike yeast cells, *B. hordei* conidiospores do not accumulate significant amounts of thiamine phosphate esters.

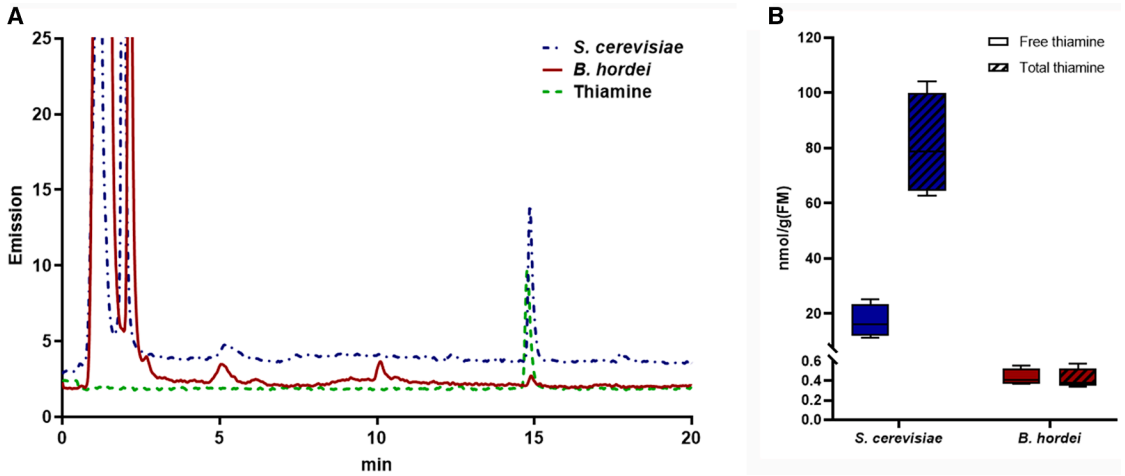


Figure 5. *B. hordei* conidiospores contain detectable levels of thiamine

(A) Chromatograms obtained during HPLC-FLD (excitation 360 nm; emission 440 nm) analysis of derivatized *B. hordei* conidiospores and yeast cell extracts. Derivatized thiamine standard was analyzed for reference.

(B) Results of quantitative analysis of four technical replicates of free and total (after phosphatase treatment) thiamine content (given in nmol per gram fresh mass [FM]) in analyzed samples.

However, it cannot be excluded that *B. hordei* produces modified phosphate esters or other polar thiamine derivatives that are resistant to phosphatase treatment.

A homolog of *Candida* DUR31 mediates thiamine uptake in *B. hordei*

As we had failed to identify any close homologs of *THI7* family genes, which encode the major thiamine transporters in *S. cerevisiae*, in the genome of *B. hordei* (see above and Figure 2), we searched for homologs of the thiamine importer termed *DUR31* that is present in several *Candida* species.[18] *C. albicans* DUR31 is a member of a family of seemingly ancient thiamine transporters, as putative orthologs of it have been recognized not only in a broad range of unicellular and filamentous ascomycetes but also in basidiomycetes and oomycetes.[18,50,51] However, this transporter is not found in species of the genus *Saccharomyces*.[18]

We identified a homolog of *CaDUR31* in the *B. hordei* genome (EnsemblFungi: *BLGH_00877*; hereafter termed *BhDUR31*). *BhDUR31* shares 55% identity with *CaDUR31* at the nucleotide level, and the respective gene products exhibit 67% similarity and 49% identity, respectively. Moreover, like *Ca*DUR31, *Bh*DUR31 has 13 predicted transmembrane domains (Figure S10). Due to the lack of reproducible transformation protocols for powdery mildew fungi, we again aimed to test the function of *Bh*DUR31 in baker's yeast. The *S. cerevisiae* CVY4 mutant strain[52] is deficient in thiamine biosynthesis through a knockout of the *Thi4* gene and severely limited in thiamine uptake by the deletion of the three thiamine importer genes *Thi7*, *Thi71*, and *Thi72* (Figure 1). In this thiamine-auxotrophic quadruple mutant, *BhDUR31* was expressed alongside an empty vector negative control and the native *S. cerevisiae Thi7* gene (positive control). Transformed CVY4 yeast cells were plated on a minimal medium that was supplemented with a range of thiamine concentrations (0–120 μM) and grown for 3 days at 28°C. At the two highest thiamine concentrations (12 and 120 μM), there was almost no growth difference between the three transformants (Figure 6), indicative of residual thiamine uptake of the yeast CVY4 cells at elevated thiamine concentrations. From 1.2 μM downward, the empty vector transformant exhibited almost no growth, whereas the *ScThi7*- and *BhDUR31*-transformed yeast strains grew similarly to the higher thiamine concentrations down to 0.12 μM, indicating complementation of the genetic defects in the CVY4 strain by these two genes. At 0.012 μM thiamine, these two strains exhibited reduced yet still visible growth, whereas, in the absence of thiamine (0 μM), only the weak outlines of the cells transferred from the liquid medium were visible (Figure 6). In summary, we found that *BhDUR31* complements the yeast CVY4 mutant strain to a similar extent as the native *ScThi7* gene. We thus conclude that *BhDUR31* encodes a functional plasma-membrane-localized thiamine transporter protein that can mediate thiamine uptake from the extracellular environment.

Most thiamine-associated genes are not regulated at the transcriptional level in *B. hordei* and *H. vulgare* in the course of fungal infection

We took advantage of published transcriptome data to explore the transcriptional regulation of thiamine-associated *B. hordei* genes (*BhPHO3* [several candidates], *BhTHI80*, *BhDUR31*, *BhTHI74*, *BhTPC1* [several candidates], and *BhTKL*; Figure 2 and Table S1) during fungal pathogenesis. We also considered the expression levels of barley thiamine-associated genes (*TenA_E*, *Th1*, *Th2*, *Thi1*, *ThiC*, *ThiM*, *Tpk1*, and *Tpk2*) as derived from the known pathway genes in *Arabidopsis thaliana*[53] (Figure 7A), which might also be subject to transcriptional regulation during the interaction with *B. hordei*. The respective transcriptomic dataset was generated based on leaf epidermal peels sampled at various time points after fungal challenge (0, 6, 18, 24, 72, and 120 h post-inoculation[54]). We noticed that on the fungal side, there was little regulation of the thiamine-associated genes (related to

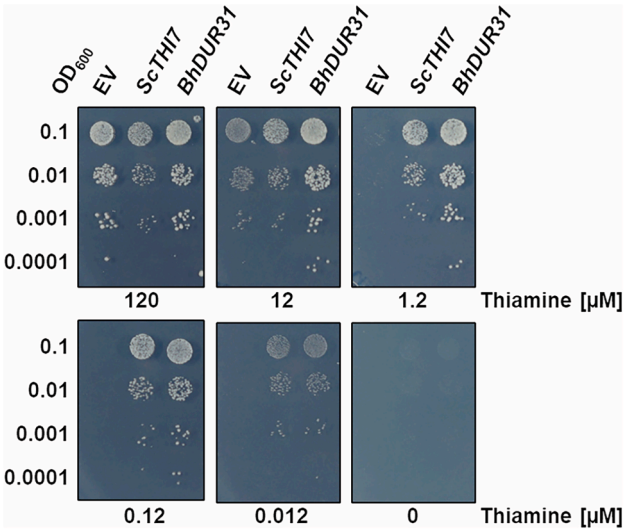


Figure 6. *B. hordei BLGH_00877* (*BhDUR31*) complements the growth defect of the *S. cerevisiae* CVY4 quadruple mutant strain on media with a low thiamine content

Cells of the baker's yeast (*S. cerevisiae*) CVY4 quadruple mutant strain (defective in *Thi4*, *Thi7*, *Thi71*, and *Thi72*) were transformed with empty vector (EV, negative control) or expression constructs encoding the native yeast Thi7p thiamine importer or the *B. hordei BLGH_00877* (*BhDUR31*) candidate gene. Depicted are various serial dilutions (OD_{600} 0.1, 0.01, 0.001, and 0.0001) of liquid yeast cultures dropped on SC plates lacking uracil and supplemented with thiamine (0–120 μM). The experiment was repeated once with the same outcome.

biosynthesis and transport) during pathogenesis. However, transcript levels of *Bh*TKL increased markedly at 72 hpi and 120 hpi (Figure 7B), possibly indicative of a higher thiamine availability in the fungus at later time points of plant infection. Also, two of the *BhPHO3* candidates (EnsemblFungi: *BLGH_06298* and *BLGH_06861*) exhibited increased transcript levels between 0 and 18 h by 13- and 3-fold, respectively. Similar to the fungal pathogen, most host genes related to thiamine biosynthesis showed unaltered expression levels in the course of fungal pathogenesis, except for barley *Thi1* and *ThiC*, which exhibited a decrease in transcript accumulation from 6 h to 18 h post-inoculation onward (Figure 7C).

DISCUSSION

Most bacterial, fungal, and plant species are prototrophic regarding thiamine as they are capable of synthesizing the vitamin from precursor molecules (Figure 1). Conversely, only a few species of this taxonomic spectrum are natural thiamine auxotrophs. Examples of the latter comprise auxotrophic bacteria such as *Rhodococcus ruber* ZM07[56] and several leaf-associated bacterial species.[57] On the fungal side, instances are the *Aspergillus fumigatus* isolate NIH4215,[58] the obligate biotrophic mycosymbionts *Tritirachium egenum*[59] and *Rhizophagus irregularis*,[60] and the plant-beneficial *Serendipita indica*.[61] Additionally, the lack of genes encoding for thiamine biosynthetic enzymes has been noticed in the genomes of various plant-infecting oomycetes (*Albugo laibachii*, *Hyaloperonospora arabidopsidis*, and *Phytophthora* species[62]). By contrast, (higher) animals have lost genes encoding key enzymes of the thiamine biosynthetic pathway early in the course of evolution, rendering this entire taxonomic group thiamine-auxotrophic. Accordingly, these species must take up the majority of thiamine in their diet, and the molecule is thus considered a vitamin (vitamin B1) for these organisms. There is, however, evidence that at least part of the thiamine required by animals might be provided by their gut microbiota.[63] Likewise, auxotrophic bacteria and fungi can acquire the essential compound from other organisms they live with in close association. For example, the thiamine auxotrophy of bacteria and fungi can be rescued when they are co-cultivated with thiamine-prototrophic bacterial species, suggesting metabolic exchange ("cross-feeding") of the vitamin and possibly other molecules across species or even kingdoms of life.[56,61,64] This can similarly be assumed for foliar bacterial communities, which were recently found to harbor a substantial proportion of thiamine-auxotrophic strains.[57]

In analogy to the rather rare documented examples of bacteria and fungi, and the common situation for (higher) animals, we and others previously noticed based on genomic information that powdery mildew fungi seemingly lost the capacity to synthesize thiamine.[4,39,65] Yet, according to the findings of the current study, at least *B. hordei* appears to be dependent on the micronutrient, which presumably like in other organisms serves as an essential co-factor for some key metabolic enzymes in these phytopathogens. The respective chain of evidence rests on (1) the conserved presence and retained functionality of the THI80 enzyme (Figure 4), (2) the presence of a functional DUR31-related thiamine transporter likely residing at the cell surface (Figure 6), (3) the detectable accumulation of thiamine in fungal conidiospores (Figure 5), (4) the conservation of predicted thiamine-binding sites in several metabolic enzymes (Data S1), and (5) the dependence of an exemplarily tested enzyme (transketolase) on the compound (Figures 3 and S7–S9). Thus, thiamine can be considered as a vitamin for *B. hordei* by definition.[66] Although we only examined the situation for the barley pathogen in detail, we postulate based on the conserved genomic features that the findings can be extrapolated to all powdery mildew fungi. Despite minor differences regarding the presence and absence of thiamine-related genes (Figure 2), which might be explained by incomplete and/or contaminated genome assemblies,[67] we argue that the overall pattern is rather consistent across the various powdery mildew species, pointing at common physiological conditions in these fungi. This would add an entire taxonomic group (*Erysiphaceae*, rank of a family[30]) to the otherwise yet relatively short list of microbial species that are known as thiamine auxotrophs.[7,56–60]

The loss of the thiamine biosynthetic pathway must have occurred early in the evolution of the powdery mildew fungi as according to BLAST searches essentially all powdery mildew species seem to lack the same spectrum of thiamine-related genes, except for *G. magnicellulatus* and the early diverged powdery mildew species *P. polyspora* (Figure 2). Reciprocal TBLASTN searches provide, however, evidence that in the case of the latter two species, these sequences likely derive from contaminating non-powdery mildew microbes (Table S2), which is a known phenomenon for the genome assemblies of

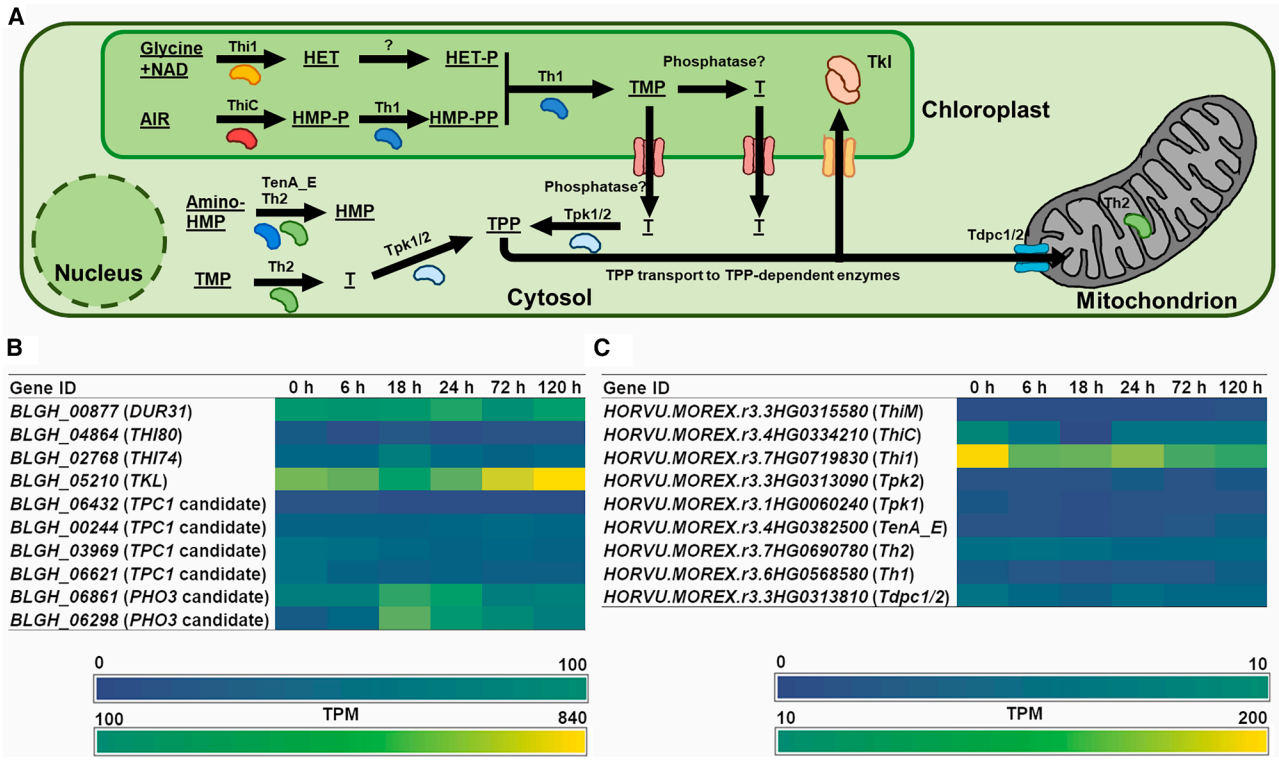


Figure 7. Transcript levels of genes involved in thiamine biosynthesis and uptake during infection of barley by *B. hordei*

(A) Proposed plant thiamine biosynthesis pathway (modified from[53]). Using NAD^+ and glycine, Thi1 synthesizes 4-methyl-5-(2-hydroxyethyl) thiazole (HET, which is further converted to 4-methyl-5-(2-hydroxyethyl) thiazole phosphate [HET-P]). Another precursor is made from aminoimidazole ribonucleotide (AIR) by Thic and Th1. This produces 4-amino-2-methyl-5-hydroxymethylpyrimidine phosphate (HMP-P), and Th1 uses these to create 4-amino-2-methyl-5-hydroxymethylpyrimidine pyrophosphate (HMP-PP). In the final step, Th1 fuses HMP-PP and HET-P to thiamine monophosphate (TMP). These processes happen in the chloroplast. How thiamine (T) or TMP is transported into the cytosol is currently unknown. In the cytosol, thiamine is converted by Tpk1 and Tpk2 to the bioactive form, thiamine pyrophosphate (TPP). Tdpc1 and Tdpc2 are mitochondrial TPP transporters. Th2 and TenA_E are involved in the recycling of thiamine precursors and derivatives.

(B and C) Published transcriptome data[54] were used to generate the heat maps to illustrate transcript levels of selected *B. hordei* (A) or *H. vulgare* (B) genes in the course of *B. hordei* infection. The average values of three replicates were taken and depicted as transcripts per million (TPM) in the course of infection (at 0, 6, 18, 24, 72, and 120 h post-inoculation), color-coded according to the corresponding color legends below the heat maps. The barley genes were identified by TBLASTN searches of the respective genes of *Arabidopsis thaliana* against the barley (cultivar Morex) genome[55] and validated by additional BLASTP analysis.

some powdery mildews.[67] These presumptive contaminants comprise mostly fungi, including two *Trichoderma* species, which are known hyperparasites of powdery mildew fungi[68] but also a soil bacterium (*Paenibacillus baekrokdamisoli*; Table S2). The *P. polyspora* genome derives from metagenomic samples collected from natural infection sites on the East Asian oak tree *Quercus serrata*,[39] providing a plausible explanation for the source of the presumptive microbial contaminations. In contrast to the powdery mildews, their closest known relative, the saprophytic fungus *Arachnopeziza araneosa*,[30] seems to feature an intact thiamine biosynthetic pathway, enabling the formation of thiamine via the branch using NAD^+, glycine, and cysteine (Figures 1 and 2). Accordingly, all obligate biotrophic powdery mildew fungi appear to have lost the capacity for thiamine biosynthesis soon after their evolutionary separation from the saprophytic fungal lineage.

Regarding the conservation of THI80, transcription factors THI3 and PDC2, proteins related to thiamine uptake, and the thiamine phosphatase PHO3, we likewise noticed a largely consistent presence/absence pattern across the powdery mildew lineage (Figure 2). A notable exception is the seeming presence of putative THI7, THI71, and THI72 proteins in *Golovinomyces magnicellatus* and *P. polyspora*; however, these BLAST hits also likely derive from microbial contaminations in the genomes of these powdery mildews (Table S2). The apparent lack of individual proteins such as THI3 and PDC2 in *E. necator*, PDC2 in *P. polyspora* as well as PDC2 and DUR31 in *P. shiraiana* are probably attributable to the incomplete and fragmented genome sequences of these powdery mildew species. Interesting to mention is the putative absence of the putative mitochondrial thiamine transporter protein THI74 in all four members of the genus *Golovinomyces* (note that the BLAST hit for *G. magnicellatus* is a putative microbial contamination; Table S2), which is hence unlikely the result of poor genome qualities. It rather suggests that the gene encoding this transporter has been lost exclusively in the genus *Golovinomyces*.

Concerning the copy number of the genes/proteins studied, we found that it is generally in a similar range for powdery mildew fungi and non-powdery mildew ascomycetes (Figure 2). According to

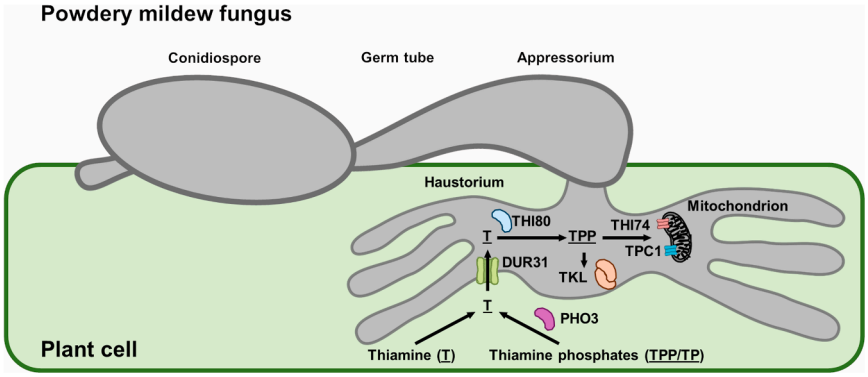


Figure 8. Graphical summary of our findings regarding the thiamine uptake pathway in *B. hordei*

The scheme shows a germinated powdery mildew conidiospore on the surface of a leaf epidermal plant cell. The fungal sporeling has a germ tube and an appressorium and has formed successfully a haustorium inside the plant host cell. Our data suggest that powdery mildews lack enzymes for the biosynthesis of thiamine (T) (Figure 2) but still have a functional uptake and conversion pathway, comprised of the DUR31 thiamine transporter (Figure 6) and the thiamine pyrophosphate kinase THI80 (Figure 4), which converts thiamine to its bioactive form thiamine pyrophosphate (TPP). We further provide *B. hordei* candidates for the extracellular acid phosphatase PHO3, which dephosphorylates thiamine phosphates (TPP and/or TP) to thiamine, and the mitochondrial TPP transporters THI74 and TPC1 (Figure 7). Most of the enzymes that use TPP as a cofactor are located in mitochondria. Our *in silico* analysis depicts the presence of the TPP binding fold in these enzymes (Table S3), and we experimentally validated the TPP dependency for transketolase (TKL; Figure 3). The most likely source of thiamine is the host plant.

our analysis, *THI80*, *THI74*, and *DUR31* seem to be single-copy genes in all ascomycetes, including the powdery mildews. *THI3* (1–3 copies) and *PHO3* (2–3 copies) are present in moderately elevated numbers in the powdery mildews, which are nevertheless lower than in the non-powdery mildew ascomycetes (2–4 and 3–6 copies, respectively; Figure 2). We detected even higher copy numbers for *PDC2* (2–98 copies) and *TPC1* (14–20 copies) in the powdery mildew fungi, which at least for *TPC1* mirrors the situation in the non-powdery mildew ascomycetes. Whether all of these code for functional *bona fide* PDC2 and TPC1 proteins remains elusive and requires further investigation.

The thiamine levels determined in cells of *S. cerevisiae* (Figure 5B) are in the range previously reported for baker's yeast and other fungi.[69–71] Compared to these values, thiamine concentrations in *B. hordei* conidiospores are low (Figure 5B). This might be a consequence of the lack of the respective biosynthetic pathway and the need for thiamine uptake from the external environment in the case of the obligate biotrophic phytopathogens. However, the root-colonizing arbuscular mycorrhizal fungus *R. irregularis* also lacks thiamine biosynthesis[60] but accumulates considerably higher levels of thiamine in its spores than the roots of its host plant, *Lotus japonicus*.[70] The discrepancy in thiamine accumulation in spores of *B. hordei* and *R. irregularis* might be explained by the higher thiamine concentrations in plant roots as compared to shoots and leaves.[72] Also, *B. hordei* conidiospores are largely metabollically inactive and presumably need only low thiamine levels to sustain spore germination and host cell entry. Finally, powdery mildews may store thiamine in forms that are not released by phosphatase treatment, leading to an underestimation of the actual thiamine levels.

We hypothesize that the thiamine required by powdery mildew fungi may largely derive from their plant hosts, which are thiamine-prototrophic. The micronutrient might be absorbed via the powdery mildew haustoria, which are in close contact with a modified version of the host plasma membrane (extrahaustorial membrane), separated from it only by the extrahaustorial space.[3,35] We speculate that the pathogen may redirect the flow of thiamine from host epidermal cells toward the fungal haustoria, e.g., with the help of effector proteins that reprogram the plant's metabolism (Figure 8). Indeed, several plant-colonizing and haustorium-forming oomycetes (*Albugo laibachii*, *Hyaloperonospora arabidopsidis*, and *Phytophthora* species) as well as the arbuscle-forming symbiont *R. irregularis* were previously found to lack genes encoding proteins related to thiamine biosynthesis,[60,62] suggesting that these species may retrieve thiamine from their plant hosts via their dedicated infection structures (haustoria and arbuscules[70]). This might be an adaptation to the high metabolic costs of thiamine biosynthesis, as THI4, a key enzyme in the pathway (Figure 1), is a so-called suicide enzyme that undergoes only a single turnover.[26,73] Experimental options to explore the source of fungal thiamine in the barley-*B. hordei* interaction further would be isotope-labeling of thiamine fed to host plants, the overexpression of thiamine-modifying enzymes such as thiamine pyrophosphokinase to trap TPP inside plant cells, or the expression of an extracellular thiaminase in the plant apoplast. The single-cell transient gene expression assay, as established for the barley-*B. hordei* interaction,[74,75] would be a possible route for the latter suggestions.

Thiamine seems an essential molecule not only for powdery mildew fungi but also for other taxonomically different phytopathogens. Thiamine auxotrophy is a well-known phenomenon in plant-pathogenic oomycetes such as *Phytophthora* species as these, similar to powdery mildew fungi, have lost the respective biosynthetic pathway.[76] In the case of *Phytophthora sojae*, a notorious soil-borne pathogen of soybean (*Glycine max*), it was found that the compound is available to the invader from both host root exudates and rhizosphere bacteria. However, myxobacteria antagonize root invasion of the oomycete by scavenging thiamine in the soybean rhizosphere via an enzyme (thiaminase I) that is secreted by bacterial outer membrane vesicles.[77] By analyzing induced mutants in genes related to thiamine biosynthesis, it was discovered that the renowned bacterial phytopathogen *Pseudomonas syringae* pv. *tomato* DC3000 requires the vitamin for survival and virulence.[78] A class of phytopathogens that retained the thiamine biosynthetic pathway are the rust fungi. Notably, these fungi seem to require high levels of vitamin B1, as transcripts of thiamine biosynthetic enzymes

are highly abundant in the haustoria of the bean rust pathogen, *Uromyces fabae*.[29]

The diversity of fungal plasma-membrane-localized thiamine transporters is quite remarkable. These cell surface transporters enable the uptake of the vitamin from the environment and, thus, supplement its biosynthesis. While the budding yeast *S. cerevisiae* uses Thi7p (Thi10p) as the primary thiamine transporter[15,16] and Thi71p and Thi72p as secondary transporters,[17] the fission yeast *S. pombe* deploys the unrelated and seemingly taxonomically restricted transporter Thi9p.[52] Other ascomycete fungi have yet other transporters, such as those related to DUR31 of *C. albicans*.[18,19] We noticed high expression levels of a gene (Ensembl Fungi: *BLGH_00877*) encoding a DUR31-related transporter in *B. hordei*, which prompted us to test this protein for its capacity to import thiamine in a yeast-based complementation assay (Figure 6). The fact that this transporter (*Bh*DUR31) is functional suggests that *B. hordei* imports thiamine from the environment, e.g., its host plant. Since also genes coding for putatively secreted PHO3-like thiamine phosphatases[79] are present in powdery mildew genomes (Figure 2) and expressed in *B. hordei* (Figure 7), thiamine phosphates (TPP and/or TP) are possibly converted to thiamine prior to its uptake by *Bh*DUR31 and its subsequent intracellular re-conversion to the bioactive form, TPP (Figure 8). Secreted *B. hordei* PHO3-like thiamine phosphatases may also enter host cells, acting as effector proteins that convert TPP to thiamine to enable its export to the extracellular environment.

We noticed that the transcript levels of most genes related to thiamine biosynthesis, uptake, or usage do not change drastically in the course of infection, except for a fungal *PHO3* candidate (EnsemblFungi: *BLGH_06298*) and host genes *ThiC* and *Thi1* (Figure 7). This is somewhat surprising, as one could have expected compensatory gene upregulation on the host side upon the establishment of a thiamine sink in the form of the fungal pathogen. However, the absence of compensatory gene regulation might be explained by excessive amounts of thiamine present in host cells, even upon colonization by the powdery mildew pathogen, which would alleviate the need for extensive compensatory regulatory changes. The downregulation of *ThiC* and *Thi1* in the early phase of infection could be interpreted as a countermeasure of the host to limit thiamine availability, as both enzymes act early in both biosynthetic branches (Figure 7). The phenomenon that plants can actively restrict nutrient availability is also called "nutritional immunity" and is, for example, well described for iron restriction.[80] Alternatively, the pathogen may manipulate the expression of *ThiC* and *Thi1* to reduce thiamine levels in host cells to levels that allow for fungal growth but negatively affect the host's defense response.

We also would have predicted elevated transcript levels on the fungal side upon the establishment of a thiamine source in the form of the plant host. The absence of such changes in gene expression indicates that the respective genes are not subject to extensive regulation at the transcriptional level. In thiamine-prototrophic species such as baker's yeast, thiamine starvation results in transcriptional upregulation of genes related to the biosynthesis of the vitamin, which is suppressed in the presence of sufficient extracellular thiamine levels.[17] Additionally, thiamine-related protein biosynthesis is often regulated at the post-transcriptional level by so-called TPP riboswitches. Riboswitches are secondary structures present in mRNAs that bind small molecules, thereby regulating the efficiency of the splicing and/or translation of the respective mRNA. In TPP riboswitches, the small molecule is TPP, allowing feedback regulation regarding the accessibility of thiamine-associated transcripts for protein biosynthesis. The principle of TPP riboswitches is established in many taxonomic groups, e.g., bacteria,[81] fungi,[18,82] algae,[83] and plants.[84] Further in-depth bioinformatic and functional analysis would be required to unravel whether TPP riboswitches also exist in the relevant transcripts of barley and *B. hordei* genes.

Limitations of the study

Given that powdery mildews, including *B. hordei*, are obligate biotrophic phytopathogens that cannot be manipulated genetically in a targeted manner, we cannot test our hypotheses by direct genetic perturbation, e.g., the (conditional) knockout of *B. hordei THI80* or *DUR31*. We further appreciate that we extrapolate findings experimentally obtained from *B. hordei* (Figures 3, 4, 5, 6, and 7) to other powdery mildew fungi that lack a similar repertoire of thiamine-biosynthesis-related genes (Figure 2). We also admit that at present we do not know the origin of thiamine that powdery mildew fungi utilize.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Ralph Panstruga (panstruga@bio1.rwth-aachen.de).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact with a completed materials transfer agreement.

Data and code availability

- Data: this paper analyzes existing, publicly available data (*B. hordei* RNA-seq data), accessible at https://www.ncbi.nlm.nih.gov/sra under BioProject ID PRJNA835302, the derived updated *B. hordei* proteome (v4.3), accessible at https://doi.org/10.1186/s13100-023-00305-6, and various fungal genomes (sources listed in Table S4). All new data reported in this paper will be shared by the lead contact upon request.
- Code: this paper does not report original code.
- Additional information: any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

R.P. conceived the study and supervised the project.
B.S. performed the *in silico* analysis.
B.S., M.F., and M.L. performed yeast assays.

J.B. and A.M.V. performed heterologous protein expression and TKL enzymatic assays.

B.S. and M.L. cloned the constructs.

B.S. collected *B. hordei* conidiospores for thiamine measurements.

M.P.B. and P.B. performed measurements of thiamine content in *B. hordei* conidiospores.

B.S. and M.F. drafted the manuscript; R.P. edited the manuscript.

All authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Escherichia coli TOP10	Thermo Fisher Scientific (Invitrogen)	C404010
Escherichia coli Rosetta	Merck (Sigma Aldrich)	70953-3
Chemicals, peptides, and recombinant proteins		
D,L-glyceraldehyde	Sigma Aldrich	G5001-500MG; CAS: 56-82-6
Gateway™ BP Clonase™ II Enzym-Mix	Thermo Fisher Scientific (Invitrogen)	11789020
Gateway™ LR Clonase™ II Enzym-Mix	Thermo Fisher Scientific (Invitrogen)	11791020
G418	Santa Cruz	sc-29065A; CAS: 108321-42-2
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Carl Roth	2316.3; CAS: 367-93-1
Lithium acetate	Carl Roth	5447.1; CAS: 546-89-4
Lithium-ß-hydroxypyruvate (LiHPA)	Sigma Aldrich	54913; CAS: 3369-79-7
Magnesium chloride	Carl Roth	2189.3; 7791-18-6
Phenole red	Sigma Aldrich	P3532-5G; CAS: 143-74-8
Polyethylene glycol (PEG)	Aldrich Chemistry	81240-1KG; CAS: 25322-68-3
Thiamine	Sigma Aldrich	T4625; 67-03-8
Thiaminepyrophosphate	Sigma Aldrich	C8754; 154-87-0
Triethanolamin	Sigma Aldrich	T9534; CAS: 637-39-8
T4 DNA ligase	New England Biolabs	M0202S
Yeast Nitrogen Base without amino acids	Formedium	CYN0405
Yeast Nitrogen Base without amino acids and thiamine	Formedium	CYN4701
Yeast synthetic drop out medium supplement without Uracil (SC)	Formedium	DSCK102
Critical commercial assays		
Protein Ark Quick Coomassie Stain	Calibre Scientific	PAR-GEN-QC-STAIN-1L
Protino Glutathione Agarose 4B beads	Macherey-Nagel	745500.10
Thrombin protease	Cytiva Global Life Sciences Solutions USA LLC	27084601
Experimental models: Organisms/strains		
Blumeria hordei (isolate K1)	Hinze et al.[85]	Field isolate
Erysiphe pisi	Spanu et al.[4]	Field isolate
Golovinomyces orontii	Spanu et al.[4]	Field isolate
Saccharomyces cerevisiae thermosensitive *thi80* mutant	Ben-Aroya et al.[46]	YOR143C, Ts747-1
Saccharomyces cerevisiae strain CVY4	Vogl et al.[52]	CVY4
Oligonucleotides		
See Table S4 for oligonucleotide sequences	This paper	Not applicable
Recombinant DNA		
pAG426GPD-ccdB	Alberti et al.[86]	Addgene #14156
pAG426GPD-Sc THI7	This paper	Not applicable
pAG426GPD-Bh DUR31	This paper	Not applicable
pDONR207	Invitrogen	Not applicable
pDONR207-Sc THI7	This paper	Not applicable
pDONR207-Bh DUR31	This paper	Not applicable

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pFA6a-KanMX4	NovoPro Bioscience Inc.	V010318
pFA6a-Sc THI80 Pro_Sc THI80 Term	This paper	Not applicable
pFA6a-Sc THI80 Pro_Sc THI80_Sc THI80 Term	This paper	Not applicable
pFA6a-Sc THI80 Pro_Bh THI80_Sc THI80 Term	This paper	Not applicable
pFA6a-Sc THI80 Pro_Go THI80_Sc THI80 Term	This paper	Not applicable
pFA6a-Sc THI80 Pro_Ep THI80_Sc THI80 Term	This paper	Not applicable
pFA6a-Sc THI80 Pro_Bh THI80D77N_Sc THI80 Term	This paper	Not applicable
pFA6a-Sc THI80 Pro_Bh THI80D106N_Sc THI80 Term	This paper	Not applicable
pGEX-2TK	Sigma-Aldrich	GE28-9546-53
pGEX-2TK-Sc TKL	This paper	Not applicable
pGEX-2TK-Bh TKL	This paper	Not applicable
Software and algorithms		
Boxshade	Albà[87]	https://junli.netlify.app/apps/boxshade/
ColabFold	Mirdita et al.[88]	ColabFold v1.5.5
Galaxy server	Galaxy Community[89]	https://usegalaxy.eu/
Interproscan	Jones et al.[90]	http://www.ebi.ac.uk/interpro/search/sequence/
PyMol	Not applicable	https://www.pymol.org/
T-Coffee	Notredame et al.[91]	https://tcoffee.crg.eu/apps/tcoffee/do:regular
Other		
Blumeria hordei proteome	Qian et al.[54]	v4.3
Blumeria hordei RNAseq dataset	Qian et al.[54]	https://www.ncbi.nlm.nih.gov/sra; BioProject ID PRJNA835302
See Table S4 for fungal genomes used in this study	This paper	Not applicable

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Powdery mildew isolates

For the propagation of the barley powdery mildew fungus *B. hordei* (isolate K1;[85,92]), one-week-old barley (cv. Lotti or Margret) plants grown in a growth chamber at 20°C and 16 h of 80-95 μmol s^{-1} m^{-2} light were inoculated and kept for one week in growth chambers (20°C /19°C day/night and 14 h of approx. 150 μmol s^{-1} m^{-2} light). Apart from *B. hordei* (isolate K1), anonymous field isolates of the pea powdery mildew pathogen *E. pisi*[4] and the Arabidopsis pathogen *G. orontii*[4] were used for the cloning of *THI80* genes.

Yeast (*S. cerevisiae*) strains

S. cerevisiae strains (Table S4) were cultivated at 28°C either in Yeast Peptone Dextrose (YPD) media with or without the antibiotic G418 or in Synthetic Complete (SC,[93]) media lacking uracil. Liquid cultures were incubated at 250 revolutions per minute (rpm) and 28°C. *S. cerevisiae* strain EBY100 (ATCC, Manassas, VA, USA) was used as the wild-type control and to clone the respective thiamine genes of yeast, strain BY4742[94] for the extraction of thiamine. *S. cerevisiae* strain YOR143C (Ts747-1) was used for the *THI80* complementation experiments and the *S. cerevisiae* CVY4 quadruple mutant strain (defective in *Thi4*, *Thi7*, *Thi71*, and *Thi72*;[52]) for the complementation assays with the thiamine transporters. *E. coli* Top10 (Thermo Fisher Scientific, Darmstadt, Germany) was utilized for cloning purposes, and *E. coli* Rosetta (Merck, Darmstadt, Germany) for protein expression (Table S4). The latter were cultivated at 37°C on LB media supplemented with suitable antibiotics.

METHOD DETAILS

In-silico analysis

The genomes of 16 powdery mildew species (19 genomes in total) and 6 ascomycetes (Table S4) were uploaded to the Galaxy server ([89]; https://usegalaxy.eu/) to create a custom BLAST library. TBLASTN searches were performed with known proteins involved in the biosynthesis and uptake of thiamine in the yeasts *S. cerevisiae* and *C. albicans* as queries. The results were filtered for the best BLAST hit(s), and the respective e-values were extracted and visualized as a heat map. The assessment of copy numbers is based on the TBLASTN results (Table S1) for each gene that (1) is present in the majority of the powdery mildew species and (2) for which a genome with a BUSCO score of $\geq$89% exists. Sequence alignments were performed with the standard algorithm for highly similar sequences (DUR31 and TKL proteins) and the ClustalW multiple sequence alignment algorithm for more distinct sequences (THI80 proteins) in T-Coffee ([91]; https://tcoffee.crg.eu/apps/tcoffee/do:regular) and visualized using BoxShade ([87]; https://junli.netlify.app/apps/boxshade/). Functional domains were predicted with InterProScan ([90,95]; http://www.ebi.ac.uk/interpro/search/sequence/). 3D structures were predicted with ColabFold (ColabFold v1.5.5: AlphaFold2 using MMseqs2;[88]) and visualized with PyMol (https://www.pymol.org/). The *B. hordei* proteome v4.3[54] was mined for the identification of putative thiamine-dependent enzymes.

Cloning

Genes from *S. cerevisiae* were cloned by polymerase chain reaction (PCR) amplification from genomic DNA, which was extracted by growing a 5-ml culture at 28°C overnight to saturation and subsequently harvested by centrifugation. The supernatant was discarded, and the cells were resuspended in residual liquid. To this cell suspension, 200 µl of glass beads, 200 µl of buffer A (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8), and 200 µl of phenol:chloroform:isoamyl alcohol (25:24:1) were added. Cells were mixed by vortexing and 200 µl of TE were mixed into it. The solution was centrifuged at 6,000 x *g* for 5 min and the aqueous phase was transferred to a new tube and 100 µl chloroform was added to the tube. The mixture was vortexed and centrifuged, and again, the upper aqueous phase was transferred to a new tube. The solution was mixed with 1 ml of 100% EtOH and centrifuged at 6,000 x *g* for 2 min. The supernatant was discarded, and the pellet was resuspended in 400 µl TE and 10 µl of 4 M ammonium acetate. Afterward, 1 ml of 100% EtOH was added, and the mixture was centrifuged for 2 min at 6,000 x *g*. Finally, the supernatant was discarded, and the pellet dried and resuspended in 50 µl of TE.

Coding sequences of genes from *B. hordei* (isolate K1), *E. pisi* (field isolate;[4]), and *G. orontii* (field isolate;[4]) were cloned by PCR amplification from cDNA. Therefore, total RNA was extracted from heavily infected plant material (barley, pea, and *Arabidopsis thaliana*, respectively) with the Macherey-Nagel Nucleo Spin RNA Plant Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. RNA was transcribed to cDNA with the SuperScript II reverse transcriptase from Invitrogen (Invitrogen, Karlsruhe, Germany) by following the manufacturer's protocol. The *Thi80* coding sequence was cloned under its native promoter and terminator via classical cloning. For this, the *THI80* promoter fragment and the vector pFA6a-KanMX4 (NovoPro Bioscience Inc., Shanghai, China) were cut with restriction endonucleases *Pvu*II and *Xma*I to create overhangs and were ligated with a T4 DNA ligase (New England Biolabs, Ipswich, Massachusetts, USA). The *THI80* terminator sequence was inserted by cutting the respective PCR fragment and vector with restriction endonucleases *Pac*I and *Asc*I. Genes of interest were inserted as PCR fragments digested with restriction endonucleases *Xma*I and *Pac*I. Plasmid extraction from *E. coli* TOP10 cells was performed using the Macherey-Nagel NucleoSpin Plasmid Kit. The resulting vectors were validated by DNA sequencing (Eurofins Genomics, Eberberg, Germany). The vector with *BhThi80* was used as a template for the site-directed mutagenesis with complementary primers that contain the indicated mutations. The vector was completely PCR-amplified with a proofreading polymerase and the PCR product was digested with restriction endonuclease *Dpn*I. The PCR product was purified with the Roche HighPure PCR clean-up micro kit and transferred into *E. coli* Top10 via heat shock transformation and the plasmid was reisolated and sequenced. Transketolase of *S. cerevisiae* and *B. hordei* were cloned by cutting the vector pAK-SS and respective PCR fragments with restriction endonucleases *Xma*I and *Eco*RI. The other constructs were generated by Gateway® (Life Technologies, Waltham, Massachusetts, USA) cloning with a modified protocol. For Gateway® BP and LR reactions, 1 µl of each (TE, ddH_2O, PCR product/entry clone, entry vector/destination vector) was mixed with 1 µl of Gateway® BP or LR clonase and incubated overnight at room temperature. Respective reactions were transformed into chemically competent *E. coli* Top10 cells via heat-shock transformation and streaked on media with appropriate antibiotics. Plasmids were again isolated from liquid cultures using the Macherey-Nagel NucleoSpin Plasmid Kit. We selected pDONR207 as the entry vector and used the Gateway®-compatible pAG426GPD-ccdB[86] as the destination vector for the expression in yeast. Correct insertion of the coding sequences of interest as well as promoter and terminator regions were validated by Sanger sequencing. Verified clones were used for further experiments. An overview of all vectors, clones, and primer sequences is provided in Table S4.

Yeast transformation

A yeast culture was incubated in YPD overnight at 28°C and 250 rpm. The 50-ml main culture was set to OD_{600} 0.2 and incubated until it reached OD_{600} 0.8 – 1. The culture was harvested by centrifugation at 3,000 *g* for 5 min. The pellet was washed once with 0.5 volume of sterile water and once with 0.01 volume of LiAc/TE (100 mM LiAC, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Of this cell

suspension, 50 µl was used per transformation reaction and mixed with 300 ng – 1 µg plasmid DNA, 50 µg high-quality sheared salmon sperm carrier DNA (boiled for 5 min at 100°C) and 300 µl sterile transformation buffer (40% PEG-4000, 100 mM LiAc, 10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The suspension was incubated at 28°C for 30 min and then a heat-shock at 42°C for 20 min was performed. Yeast cells were plated either on YPD medium with G418 (0.2 g/l) or SC media lacking the respective amino acid(s) for selection. Plates were incubated for two to three days at 28°C.

Drop test for yeast growth

Yeast cultures were grown overnight at 28°C and 250 rpm and harvested at 1,500 g for 1 min. The cells were washed once with 1 ml of sterile water and resuspended in 500 µl of sterile water. The OD_{600} was measured and set to OD_{600} 1 or 0.1 in the highest dilution. A 10-fold dilution series until an OD_{600} of 0.001/0.0001 was performed. Yeast suspensions were dropped on selective media and were grown for three days at 28 or 37°C.

Thiamine extraction from spore material and yeast

Conidiospores of *B. hordei* were collected by vacuuming heavily infected barley plants with an in-house modified vacuum cleaner. Different-sized meshes (100 µm, 64 µm, and 6 µm) in multiple small chambers were put in front of the nozzle to separate dirt from spore material. About 200 mg of *B. hordei* spores or wild-type yeast cells (strain BY4742, grown in YPD medium) were homogenized with sand in 1.2 ml of 10 mM HCl using a vortex and incubated for 30 min at 80°C. Samples were centrifuged and the supernatants were collected for further analysis. To analyze total thiamine, aliquots of 50 µl extract were mixed with 35 µl of 0.5 M CAPS/NaOH buffer (pH 9.5), 5 µl of 10 mM $MgCl_2$ and 10 µl of 0.5 U/µl phosphatase from bovine intestinal mucosa (Sigma-Aldrich, Taufkirchen, Germany), and incubated at 37°C for 70 min. Thiamine was analyzed after conversion to the corresponding thiochrome.[49,96] For this purpose, 100 µl aliquots of crude extracts as well as phosphatase reactions were mixed with 100 µl of 1M NaOH and 12.5 µl of 0.1% $K_3[Fe(CN)]_6$.

HPLC-FLD measurement

Reaction mixtures were subjected to HPLC analysis on an Agilent 1200 HPLC System equipped with a fluorescence detector (Agilent, Waldbronn, Germany). Samples were analyzed on a Synergi Polar-RP column (100×2,0 mm, 2,5 µm; Phenomenex, Aschaffenburg, Germany) with 0.1% trifluoroacetic acid as solvent A and 98% acetonitrile/0.1% trifluoroacetic acid as solvent B at a flow rate of 0.25 ml/min at 16°C (gradient of solvent A: 95% at 0 min; 95% at 5 min; 75% at 13 min, 0% at 14 min). The peak of derivatized thiamine was identified by excitation at 360 nm and emission at 440 nm by referring to an authentic thiamine standard (Sigma-Aldrich) that was derivatized according to the same protocol. Thiamine concentrations were calculated based on the comparison of peak areas in extracts with those obtained during HPLC analyses of known amounts of the derivatized standard.

Heterologous protein production

Recombinant *S. cerevisiae* and *B. hordei* transketolase enzymes were produced in *E. coli* Rosetta cells transformed with pAK-SS_GST-*Sc*TKL, pAK-SS_GST-*Bh*TKL or the empty pAK-SS_GST plasmid as a negative control, respectively. Therefore the *E. coli* main cultures were grown for four to five hours at 37°C and subsequently supplied with isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration 1 mM) to induce the production of the homologous protein. The cultures were then grown for approx. 16 hours at 28°C and on the next day the cells were harvested and resuspended in protein purification buffer (Table S4). The cells were then lysed by sonication (two times for 1 min with 60% cycle and 40% power; Sonopuls GM 70, Bandelin electronic GmbH & Co. KG, Berlin, Germany), pelleted by centrifugation for 30 min at 4°C and 20,000 x *g* and the supernatants were kept for purification of the soluble proteins. The proteins were purified using the Macherey-Nagel Protino Glutathione Agarose 4B beads (Macherey-Nagel, Düren, Germany), and the GST tag was subsequently removed with Thrombin protease (Cytiva Global Life Sciences Solutions USA LLC, Marlborough, MA, USA).

SDS polyacrylamide gel electrophoresis (SDS-PAGE)

For SDS-PAGE, the gel components were mixed according to Table S4. Proteins were separated on the polyacrylamide gels for 50 to 60 min at 175 V, and the gel was subsequently stained in Protein Ark Quick Coomassie Stain (Calibre Scientific, Rotherham, UK). Protein bands were detected using a Chemidoc XRS+ system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

pH-based assay for transketolase activity

Transketolase activity was examined in a pH-based assay adapted from.[45] Of the respective recombinantly produced enzyme, 12 µg were mixed with 2 mM TEA buffer (2 mM triethanolamine, pH 7.5), 0.028 mM phenol red, 9 mM $MgCl_2$, 2,4 mM TPP, 50 mM D,L-glyceraldehyde and 50 mM lithium-ß-hydroxypyruvate (LiHPA) in a total volume of 250 µl. The final addition of the LiHPA was used to start the reaction, and the increase of pH was measured as the change in OD_{560} in a transparent 96-well plate with a Tecan infinite M200 plate reader (Tecan Trading AG, Switzerland) over 20 h. Additional controls without the cofactors ($MgCl_2$, TPP) and without the

keto-group donor (LiHPA) were included to examine the specificity of the reaction. For every composition, there was a blank sample excluding the protein component to account for a natural shift of the pH, e.g. by reaction with air oxygen during the assay. As a high amount of protein was needed to include all controls, the assay was conducted with only one technical replicate, and the experiment was repeated independently three times. Due to a quick shift towards a lower pH after mixing all components, the 1-h timepoint was set to zero and the enzymatic activity was plotted as a difference from this value over time (ΔOD_{560}).

QUANTIFICATION AND STATISTICAL ANALYSIS

There are no quantification or statistical analyses to include in this study.