A horizontally transferred cyanase gene in the spider mite *Tetranychus urticae* is involved in cyanate metabolism and is differentially expressed upon host plant change

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**A B S T R A C T**

The genome of the phytophagous two-spotted mite *Tetranychus urticae* was recently sequenced, representing the first complete chelicerate genome, but also the first genome of a highly polyphagous agricultural pest. Genome analysis revealed the presence of an unexpected high number of cases of putative horizontal gene transfers, including a gene that encodes a cyanase or cyanate lyase. In this study we show by recombinant expression that the *T. urticae* cyanase remained functionally active after horizontal gene transfer and has a high affinity for cyanate. Cyanases were also detected in other plant parasitic spider mites species such as *Tetranychus evansi* and *Panonychus citri*, suggesting that an ancient gene transfer occurred before the diversification within the Tetranychidae family. To investigate the potential role of cyanase in the evolution of plant parasitic spider mites, we studied cyanase expression patterns in *T. urticae* in relation to host plant range and cyanogenesis, a common plant defense mechanism. Spider mites can alter cyanase expression levels after transfer to several new host plants, including the cyanogenic *Phaseolus lunatus*. However, the role of cyanase is probably not restricted to cyanide response, but likely to the plant nutritional quality as a whole. We finally discuss potential interactions between cyanase activity and pyrimidine and amino acid synthesis.

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1. Introduction

Mites (Arthropoda: Chelicerata) colonize a diverse range of habitats. While many live freely in the soil or in water, others are parasitic on animals, or feed on plants and fungi. Phytophagous mites primarily belong to the Prostigmata suborder (Acari: Acariformes) and all feed by exuding plant cell fluid using stylet-like mouthparts (Walter and Proctor, 1999). Within the group of plant parasites, the two-spotted spider mite *Tetranychus urticae* is the most polyphagous mite species, feeding on over 1000 different plant species (Migeon and Dorkeld, 2010), a feature unique amongst arthropods. The mite is considered a serious agricultural pest. Genome analysis revealed the presence of an unexpected high number of cases of putative horizontal gene transfers, including a gene that encodes a cyanase or cyanate lyase. In this study we show by recombinant expression that the *T. urticae* cyanase remained functionally active after horizontal gene transfer and has a high affinity for cyanate. Cyanases were also detected in other plant parasitic spider mites species such as *Tetranychus evansi* and *Panonychus citri*, suggesting that an ancient gene transfer occurred before the diversification within the Tetranychidae family. To investigate the potential role of cyanase in the evolution of plant parasitic spider mites, we studied cyanase expression patterns in *T. urticae* in relation to host plant range and cyanogenesis, a common plant defense mechanism. Spider mites can alter cyanase expression levels after transfer to several new host plants, including the cyanogenic *Phaseolus lunatus*. However, the role of cyanase is probably not restricted to cyanide response, but likely to the plant nutritional quality as a whole. We finally discuss potential interactions between cyanase activity and pyrimidine and amino acid synthesis.

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active cyanases have exclusively been reported for bacteria, fungi and plants (Elleuche and Poeggeler, 2008; Johnson and Anderson, 1987; Qan et al., 2011) and cyanases were thought to be absent in animals. However, genes homologous to bacterial cyanase genes have been recently uncovered in the genomes of nematodes (Haegeaman et al., 2011; Opperman et al., 2008), but it remains unclear whether these encode active and functional proteins. Cyanases (also known as cyanate hydrolases or cyanate lyases; EC 2.2.1.104) catalyze the decomposition of cyanate into ammonia and carbon dioxide in a bicarbonate-dependent and water independent reaction: \( \text{NCO}^- + \text{HCO}_3^- + 2\text{H}^+ \rightarrow 2\text{CO}_2 + \text{NH}_3 \) (Johnson and Anderson, 1987). In bacteria, cyanases have diverse physiological functions, ranging from cyanate detoxification and production of \( \text{NH}_3 \) as an alternative N-source, to the production of \( \text{CO}_2 \) for carbon fixation in photosynthetic cyanobacteria (Anderson and Little, 1985; Luque-Almagro et al., 2008; Miller and Espie, 1994; Suzuki et al., 1996; Walsh et al., 2000). Due to its reactivity with nucleophilic groups in proteins, cyanate is toxic to organisms (Stark, 1965), therefore cyanases could play an important function in detoxification by lowering cyanate cell concentration in cyanate rich environments (Guilloton and Karst, 1987b). The biochemical and physiological functions of cyanase in eukaryotic fungi and plants are far less understood. In the filamentous fungus \( \text{Sordaria macrospora} \), cyanase is transcriptionally regulated by arginine and cyanate, and knock-out mutants showed an increased sensitivity to cyanate in the growth medium (Elleuche and Poeggeler, 2008). A similar role in cyanate detoxification was also documented for the plants \( \text{Arabidopsis thaliana} \) and \( \text{Oryza sativa} \) (Qan et al., 2011), but the role of cyanase in plants might be more complex and is not fully understood (Guilloton et al., 2002). The main substrate for cyanase (cyanate) can be formed by oxidation of hydrogen cyanide (HCN), a known defense molecule in cyanogenic plants that inhibits mitochondrial respiration (Solomonson, 1981). Over 2500 plant species are cyanogenic and release toxic HCN from a non-toxic glycoside or lipid precursor upon attack by herbivores (Gleadow and Woodrow, 2002; Poulton, 1990). Both the amount of the non-toxic cyanogenic precursors present in a given plant tissue (cyanogenic potential; HCNp) as well as the release of toxic HCN of these precursors per unit of time (cyanogenic capacity; HCNc) are ecologically important parameters (Ballhorn et al., 2009). In response to this common plant defense mechanism, some insect herbivores have evolved a specialized \( \beta \)-cyanoalanine synthase dependent detoxification pathway (Zambonlini et al., 2008). In bacteria however, oxidation of HCN to cyanate by cyanide mono-oxygenases has been reported as a first step in detoxification (Ebb, 2004; Gupta et al., 2010). In this study, we focus on the cyanase of the spider mite \( \text{T. urticae} \), identify homologous proteins in the related spider mites \( \text{Tetranychus evansi} \) and \( \text{Panonychus citri} \) and reconstruct phylogenetic relationships between cyanases of diverse origin. To investigate the potential functional importance of this lateral gene transfer, the cyanase of \( \text{T. urticae} \) was cloned and recombinantly expressed in \( \text{E. coli} \) and its potential to metabolize cyanate was characterized in vitro. By quantifying mRNA levels in spider mites feeding on different plants, we investigated whether cyanase expression in vivo is correlated with host plant range and plant cyanogenesis.

2. Material and methods

2.1. Plants and spider mites

The \( \text{T. evansi} \) strain was originally collected from \( \text{Solanum niger} \) in Crete, Greece in 2010 and was kept in the laboratory on potted \( \text{Solanum lycopersicum} \) L. cv 'Moneymaker'. The two laboratory \( \text{T. urticae} \) strains LS-VL and London were previously described (Grbic et al., 2011; Van Leeuwen et al., 2006) and used respectively for gene expression analysis in different life stages and on different host plants. Both \( \text{T. urticae} \) strains were maintained in the laboratory on potted \( \text{Phaseolus vulgaris} \) L. cv 'Prelude'. For \( \text{T. urticae} \) gene expression analysis after host plant change, the following plants were used: cyanogenic lima bean (\( \text{Phaseolus lunatus} \) L.) genotypes 8078 and 8079 (Ballhorn et al., 2006, 2005), acyanogenic snap bean (\( \text{P. vulgaris} \) L. cv 'Prelude'), tomato (\( \text{Lycopersicum} \) L cv 'Moneymaker'), cotton (\( \text{Gossypium} \) sp.) and burclover (\( \text{Medicago truncatula} \) L.). Approximately 250 adult female spider mites (1–3 days old) were collected from \( \text{P. vulgaris} \) and placed on the new host in triplicate. Mites were collected from the new host after 24 h and after developing for 6 consecutive generations. To minimize the variation in the cyanogenic character of the two \( \text{P. lunatus} \) accessions due to age and/or leaf size (Ballhorn et al., 2005), one-week-old plants of \( \text{P. lunatus} \) and \( \text{P. vulgaris} \) were simultaneously offered on a weekly basis. Wild \( \text{T. urticae} \) populations were collected from cherry (\( \text{Prunus avium} \) L. cv 'Kordia'), elderberry (\( \text{Sambucus nigra} \) L.) and cucumber (\( \text{Cucumis sativus} \) L.) in the East Flanders province (Belgium) in 2011 and maintained on the original host for at least one generation in the laboratory before use in gene expression experiments. All plants and spider mites used in this study were reared or maintained in climatically controlled rooms at 26 °C, 60% RH and 16:8 h light:dark photoperiod.

2.2. Identification and characterization of cyanase sequences in \( \text{T. urticae}, \text{T. evansi} \) and \( \text{P. citri} \)

The \( \text{T. urticae} \) cyanase gene (tetur28g02430) was previously uncovered in the \( \text{T. urticae} \) genome (Grbic et al., 2011, http://bioinformatics.psb.ugent.be/webtools/bogas/overview/-Tetur). The homologous \( \text{T. evansi} \) cyanase sequence was identified in this study by PCR and cloning. RNA was extracted using the RNasey MiniKit (Qagen) from 150 female adult \( \text{T. evansi} \) mites followed by DNase treatment (Turbo DNA-free kit, Ambion). Of each sample, 2 μg RNA was used for first strand cDNA synthesis with the Maxima First Strand cDNA synthesis kit for RT-PCR (Fermentas Life Sciences). DNA was collected from 400 female adults by phenol–chloroform extraction and ethanol precipitation and finally resolved in 50 μl Tris—EDTA (Sigma—Aldrich, Belgium), as previously described by Van Leeuwen et al. (2008). All PCR reactions were performed with a Biometra Thermocycler Professional (Westburg, Leusden, The Netherlands) in 50 μl reaction volume with 38.2 μl of template cDNA/DNA and 5 μl PCR buffer (Invitrogen), 2 mM MgCl2, 0.2 mM dNTP (Invitrogen), 0.2 μM of each primer, 1.5 μl of template cDNA/DNA and 0.3 μl of Taq polymerase (Invitrogen). The coding sequence for \( \text{T. evansi} \) cyanase was picked up by primer pair 28g02430_Te (Table 1). Based on the retrieved \( \text{T. evansi} \) cDNA sequence, primer

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5′−3′)</th>
<th>( T_m )</th>
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<tbody>
<tr>
<td>28g02430_Te</td>
<td>F: CATAGAATGAGAATTTCATACGTTATT</td>
<td>49 C</td>
</tr>
<tr>
<td></td>
<td>R: CTTCACTTTAATGCTTCTTCTTTAGTA</td>
<td>55 C</td>
</tr>
<tr>
<td>28g02430_Te-intron</td>
<td>F: TCTGGACTGATGACGTTGCAAAACC</td>
<td>54 C</td>
</tr>
<tr>
<td></td>
<td>R: GCTCATCTTCTGCATATCC</td>
<td>54 C</td>
</tr>
<tr>
<td>28g02430_Tu</td>
<td>F: CATGATGAATGAGAATTTCATACGTTATT</td>
<td>55 C</td>
</tr>
<tr>
<td></td>
<td>R: CTTCACTTTAATGCTTCTTCTTTAGTA</td>
<td>55 C</td>
</tr>
<tr>
<td>Actin_qPCR</td>
<td>F: GCCATCTGCTCCGGATGGTCTGTTGTTGCTTGGCT</td>
<td>55 C</td>
</tr>
<tr>
<td></td>
<td>R: TCTCCGAACTTTCTCGCTTACGCAACC</td>
<td>55 C</td>
</tr>
<tr>
<td>Ubiquitin_qPCR</td>
<td>F: GCTCTCCCTGCTGATTAGTCG</td>
<td>55 C</td>
</tr>
<tr>
<td></td>
<td>R: TGGATTGTCGGCCTTGAAGGC</td>
<td>55 C</td>
</tr>
<tr>
<td>Cyanase_qPCR</td>
<td>F: TTACAGCTCATCAGCAGG</td>
<td>55 C</td>
</tr>
<tr>
<td></td>
<td>R: CATGTTAATGCCCCTGTAATA</td>
<td>55 C</td>
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pair 28g02430_Te-intron (Table 1) was designed and used to amplify and sequence T. evansi introns. All reactions were run with following conditions: initial denaturation at 94 °C for 2 min, followed by 35 cycles of 94 °C for 20 s, Tc (Table 1) for 50 s, 72 °C for 1 min 30 s and a final extension at 72 °C for 5 min. PCR products were purified using the Cycle Pure Kit (Omeba Biotek) and cloned into the pJET1.2/blunt vector (Fermentas). After transformation in DH5α E. coli cells, plasmid DNA was purified with the Plasmid Mini Kit (Omeba Biotek) and sequenced by LGC Genomics using the pJET1.2 forward and reverse sequencing primers.

The P. citri cyanase sequence was identified through a tBLASTn search (Altschul et al., 1997) against the published transcriptome dataset of P. citri (Liu et al., 2011) using the T. urticae cyanase as query. The presence of signal peptides was predicted by SignalP 4.0 (Petersen et al., 2011) and the similarity between protein sequences was calculated using MatGAT (Campanella et al., 2003).

2.3. Phylogenetic analysis of cyanase

Using the unique catalytic C-terminal domain of cyanase (IPR003712) and the T. urticae cyanase protein as query, tBLASTn-and BLASTp-searches were conducted in the NCBI database and diverse genome portals (see Table S1), resulting in a set of cyanase sequences. Cyanase protein sequences were chosen from each important clade. All protein sequences were obtained from NCBI, except for the Meloidogyne incognita and Meloidogyne hapla sequences that were retrieved from genome portals (http://www.inra.fr/meloidogyne_incognita) and (http://www.pncg.org/cbnph/) respectively (see Table S2 for accession numbers and corresponding abbreviations). An amino acid sequence alignment was constructed using MUSCLE (Edgar, 2004). ProtTest (Abascal et al., 2005) was employed to determine an appropriate model of amino acid replacement. ProtTest supported the LG amino acid replacement matrix (Le and Gascuel, 2008), with its default amino acid frequencies and a category of invariant sites (Gu et al., 1995). The variability of rates across sites was incorporated using a discrete gamma distribution of rates (Yang, 1993). Two phylogenic trees were constructed based on the MUSCLE alignment using two different maximum likelihood (ML) methods. One tree was constructed with Treefinder (version 2008) (Jobb et al., 2004). Edge support (LR-ELW) was calculated using 10,000 pseudoreplicates. Additionally, PhyML was used (Guindon and Gascuel, 2003) to conduct a ML search. The tree topology search was conducted by taking the best of NNLS & SPRs and with 5 random starting trees. The statistical significance was assessed with bootstrap analysis using 100 replications. MEGA4 (Tamura et al., 2007) was used to visualize the trees.

2.4. Quantification of plant cyanogenic potential and leaf soluble protein

Leaf cyanogenic potential (HCNp; maximum amount of cyanide that can be released from preformed cyanogenic precursors in a given tissue) of lima bean (P. lunatus) and snap bean plants (P. vulgaris) was analyzed by complete enzymatic degradation and subsequent quantitative measurement of released cyanide (Ballhorn et al., 2005). For hydrolysis of cyanogenic glycosides in leaf extracts, we used β-glucosidase isolated from rubber tree (Euphorbiaceae: Hevea brasiliensis), which is specific for the cyanogenic glycosides linamarin and lotaustralin also occurring in lima bean (Ballhorn et al., 2006). After 20 min of incubation at 30 °C in gas-tight glass vessels (Thunberg-vessels), the released cyanide was spectrophotometrically measured at 585 nm (Genesys 20, Thermo Spectronic, Madison, WI, USA) using the Spectroquant cyanide test (Merck, Darmstadt, Germany). The leaf soluble protein concentrations of the P. lunatus and P. vulgaris genotypes were determined following Bradford (1976). To test for significant differences in HCNp and soluble protein concentration among leaves of P. vulgaris and P. lunatus plants we applied one-way ANOVAs followed by least significant difference (LSD) post hoc analyses. All statistical analyses were carried out using SPSS 13 (SPSS for Windows, SPSS Inc., Chicago, USA).

2.5. Gene expression of cyanase in T. urticae

All quantitative gene expression experiments were conducted with three biological replicates. RNA was extracted and reversely transcribed to cDNA as described above. For stage specific expression analysis, 100 adults, 200 nymphs and 1000 larvae were pooled per biological replication. For gene expression analysis of spider mite strains feeding on different host plants, 75 adult female mites (1–3 days old) were used for each replicate. Gene specific qPCR primers were designed with Primer 3 (http://frodo.wi.mit.edu/) for the target gene (cyanase) and control genes (actin and ubiquitin). Primer sequences are listed in Table 1.

All qPCR reactions were conducted with a thermal cycler Mx3005P (Stratagene). Reactions had two technical replicates and were prepared using the Maxima SYBR Green qPCR Master Mix following the manufacturer’s instructions (Fermentas Life Sciences). No-template-controls were used to rule out possible sample contaminations. A final melt-curve step was included post-PCR (ramping from 95 °C to 55 °C) by 1 °C every 2 s to confirm the absence of non-specific amplification. A standard curve comprising four different pooled cDNA dilutions (ranging from the cDNA equivalent of 50 ng RNA to 0.4 ng RNA) was used for the calculation of the amplification efficiency of each primer pair. The resulting efficiencies were used in further calculations. The Ct values of the two control genes (actin and ubiquitin) were used for normalization. Analysis of the qPCR results was performed using the method as described in Pfaffl (2001), producing relative expression values of the target gene. Significant differences in gene expression of the target gene were tested with pairwise fixed reallocation randomization (Pfaffl et al., 2002).

2.6. T. urticae cyanase cloning and protein preparation

The cyanase of T. urticae was isolated by PCR from cDNA derived from the London strain using primer pair 28g02430_Tu (Table 1) as described above. The forward and reverse primer respectively inserted a Ndel site and a Xhol site. The resulting PCR product was originally cloned into the pJET1.2/blunt vector (Fermentas) and was subcloned using the inserted restriction sites into the pET-16b vector (Novagen). The resulting construct was transformed in E. coli bacterial cells. Transformed cells were grown at 30 °C in 1 L LB medium containing 100 µg/ml ampicillin. When the bacterial culture reached an absorbance of 600 nm of 0.5, the protein synthesis was induced by addition of 0.05 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Four hours after induction, the cells were harvested by centrifugation at 10,000 g for 10 min at 4 °C. Different bacterial expression strains were tested (JM109 (DE3), BL21 (DE3), BL21 (DE3) Rare, BL21 (DE3) pLYS) in preliminary experiments, but only BL21 (DE3) pLYS was finally chosen for expression of recombinant His-tagged cyanase. After harvesting, BL21 (DE3) pLYS cells were re-suspended and sonicated in buffer A: 50 mM sodium phosphate, pH 7.0, 3 mM sodium azide, 5 mM imidazole, 1 mM DTT and 5% glycerol. After sonication, the cell suspension was centrifuged at 20,000 g for 30 min at 4 °C. Soluble fractions containing the His-tagged cyanase, were loaded on a Ni2+–NTA affinity column (GE Healthcare) and after extensive washes with buffer A, the His- tagged protein was eluted with buffer B: 50 mM sodium phosphate,
pH 7.0, 3 mM sodium azide, 400 mM imidazole, 1 mM DTT and 5% glycerol. Finally the eluted protein was dialyzed against buffer C: 50 mM sodium phosphate, pH 7.0, 1 mM sodium azide, 1 mM DTT and 5% glycerol and was kept at -80 °C.

Polypeptides were separated first by 15% poly-acrylamide SDS-PAGE and then transferred on nitrocellulose membrane for western blotting. His-tagged cyanase was detected specifically with 2×His used in 1/1000 dilution (Qiagen, Tetra-His antibody).

2.7. Cyanase activity assay

Enzyme assays for measuring recombinant cyanase activity were based on the quantification of the reaction end product (ammonia) in the reaction solution and were carried out according to Anderson and Little (1985). All reagents were purchased from Sigma–Aldrich (Belgium). Solutions were prepared freshly and the purified cyanase protein was defrosted on ice. A preliminary experiment confirmed that Michaelis– Menten kinetics conditions were met under the assay conditions. The standard assay medium contained 3 mM sodium bicarbonate and 2 mM potassium cyanate in a 50 mM potassium phosphate buffer at pH 7.6 with 300 ng protein. Speciﬁc assay conditions ranged respectively from 22 and 55 °C and incubated for 11 min at 37 °C. The reaction was started by adding the second substrate (cyanate or bicarbonate) in a 3 mM sodium bicarbonate and 2 mM potassium cyanate base (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) as specific controls to assure no potential interference of the presence of NH_3 in protein preparations. Non-enzyme control reactions were immediately stopped after adding sodium chloride, and the color reagents dichloroisocyanurate and sodium salicylate (Anderson and Little, 1985). All reagents were determined with Lineweaver–Burk plots and substrate concentrations ranging from 4 mM to 0.125 mM for cyanate and 3–0.125 mM for bicarbonate. Identical protein preparations of bacterial cells transformed with the empty pET-16b vector were used as controls to assure no potential interference of E. coli endogenous cyanase was present in protein preparations. Specific activities were expressed as μmol NH_3 produced per minute and per μg protein.

3. Results

3.1. Identification and characterization of cyanase sequences in T. urticae, T. evansi and P. citri

The predicted gene structure of T. urticae cyanase, including two introns of 85 and 65 bp, was conﬁrmed by sequencing several cDNA and DNA derived clones. The corresponding full protein sequence was 160 amino acids long with a predicted molecular mass of 18.26 kDa. The sequence did not contain any predicted signal peptide. The T. urticae cyanase protein showed an average similarity of 56%, 55%, 54%, 52% and 49% to the respective sequences of cyanobacteria, fungi, plants and nematodes that were used in the phylogenetic analysis (Table S2). Sequences showed overall high conservation in the C-terminal catalytic site (Fig. 1). Using cDNA PCR and cloning, we also identiﬁed a (partial) cyanase sequence in T. evansi which was 432 bp in length and coded for 144 amino acids. By DNA PCR and cloning, the intron splice sites observed in the T. urticae cyanase were shown to be conserved in T. evansi (Fig. S1). The two introns of T. evansi cyanase were 81 and 64 bp long and showed 77.6 and 78.5% similarity to the respective introns in T. urticae. The (partial) P. citri cyanase sequence was procured from the raw data-set of a previously published transcriptome (Liu et al., 2011), and was 139 amino acids long. All spider mite sequences were recognized by the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) as cyanases and all three sequences exhibited the three conserved residues (Arg96, Glu99 and Ser122, numbering according to cyanases and all three sequences exhibited the three conserved residues (Arg96, Glu99 and Ser122, numbering according to cyanases and all three sequences exhibited the three conserved residues (Arg96, Glu99 and Ser122, numbering according to cyanases and all three sequences exhibited the three conserved residues (Arg96, Glu99 and Ser122, numbering according to cyanases and all three sequences exhibited the three conserved residues (Arg96, Glu99 and Ser122, numbering according to E. coli cyanase) (Fig. 1). Based on the complete cyanase sequence of T. urticae, it was predicted that 90.6% and 87.4% of the complete coding gene sequence of respectively T. evansi and P. citri were retrieved. The T. urticae cyanase shared an average 81.8% similarity with the two other spider mite cyanases.

3.2. Phylogenetic analysis

Database searches revealed that within fungi, the cyanase gene only occurred in Dikarya, but was common in both Ascomycota and Basidiomycota. Both divisions of Viridiplantae (Streptophyta and Chlorophyta) and some Chromalveolata (algae) harbored the gene. Cyanase was found in a wide range of bacteria, including gram negative, gram positive and cyanobacteria. Within the phylum Proteobacteria, the cyanase gene was detected in Alpha-, Beta- as well as Gamma-proteobacteria. Cyanase was not present in cyanobacteria, bacteria, fungi, plants and nematodes that were used in the phylogenetic analysis (Table S2). Sequences showed overall high conservation in the C-terminal catalytic site (Fig. 1). Using cDNA PCR and cloning, we also identiﬁed a (partial) cyanase sequence in T. evansi which was 432 bp in length and coded for 144 amino acids. By DNA PCR and cloning, the intron splice sites observed in the T. urticae cyanase were shown to be conserved in T. evansi (Fig. S1). The two introns of T. evansi cyanase were 81 and 64 bp long and showed 77.6 and 78.5% similarity to the respective introns in T. urticae. The (partial) P. citri cyanase sequence was procured from the raw data-set of a previously published transcriptome (Liu et al., 2011), and was 139 amino acids long. All spider mite sequences were recognized by the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) as cyanases and all three sequences exhibited the three conserved residues (Arg96, Glu99 and Ser122, numbering according to cyanases and all three sequences exhibited the three conserved residues (Arg96, Glu99 and Ser122, numbering according to cyanases and all three sequences exhibited the three conserved residues (Arg96, Glu99 and Ser122, numbering according to cyanases and all three sequences exhibited the three conserved residues (Arg96, Glu99 and Ser122, numbering according to cyanases and all three sequences exhibited the three conserved residues (Arg96, Glu99 and Ser122, numbering according to E. coli cyanase) (Fig. 1). Based on the complete cyanase sequence of T. urticae, it was predicted that 90.6% and 87.4% of the complete coding gene sequence of respectively T. evansi and P. citri were retrieved. The T. urticae cyanase shared an average 81.8% similarity with the two other spider mite cyanases.

![Fig. 1. Alignment of the catalytic C-terminal regions of cyanases from bacteria, fungi, plants and Metazoa. Amino-acid residues conserved in all regions are in white font on a black background, residues conserved in at least 7 sequences in white font on a dark gray background and residues conserved in at least 5 sequences are shaded in light gray. The asterisks indicate the three predicted catalytic residues from E. coli (Walsh et al., 2000). Table S2 lists the corresponding accession numbers and organisms of the used abbreviations.](image-url)
and *Photorhabdus* bacteria. These bacteria are pathogens and symbionts of insects and two nematode genera (*Heterorhabditis* sp. and *Steinernema* sp.) respectively (Goodrich-Blair and Clarke, 2007). Within Metazoa, we only detected cyanase sequences in nematodes and spider mites. Using the nematode genome portals (Table S1), we identified two cyanase genes in the *M. incognita* genome (278 and 430 amino acids) and incorporated the shortest cyanase protein in the phylogenetic reconstruction. The cyanase gene of *M. hapla* had a length of 254 amino acids. In contrast, all other cyanase proteins in this analysis were shorter than 200 amino acids. Plants, fungi and nematode cyanases had introns of various lengths and number, and no conserved intron positions, splice cites nor intron phases were detected between phylogenetic groups (Fig. S1). None of the cyanase proteins showed a predicted signal peptide and are consequently considered cytoplasmatic enzymes. The alignment of the catalytic C-domain shown in Fig. 1 demonstrated high conservation amongst all phyta for residues reported as necessary for catalytic activity in *E. coli* (Walsh et al., 2000).

The reconstruction of the phylogenetic relationships among the diverse cyanases is depicted in Fig. 2. The two ML methods produced trees with identical topologies. For each ML method, the spider mite cyanase proteins group together with high node support values. The ancestral spider mite cyanase shares a common ancestor with the cyanases of plants, fungi and cyanobacteria, but a detailed history of horizontal transfer cannot be deduced. In contrast, the nematode cyanases cluster with the large group of bacterial cyanases. Remarkably, the cyanase of the Actinobacterium *Frankia* sp. groups together with nematode sequences with high bootstrap support (Treefinder: 93%, PhyML: 89%), which could suggest that nematode cyanases originate from a horizontal transfer from bacteria.

3.3. Plant cyanogenic potential and soluble protein concentration

The cyanogenic potential of leaves showed highly significant differences between the different *Phaseolus* species and genotypes (according to one-way ANOVA: $F_{2,57} = 4142.79$, $P < 0.0001$) (see Fig. 3). Whereas *P. vulgaris* showed HCNp near the detection limit, both *P. lunatus* genotypes (8079 and 8078) were clearly cyanogenic. Post hoc analysis (LSD, $P < 0.05$) revealed that leaves of lima bean plants of the genotype 8079 showed a significantly lower HCNp than genotype 8078 and that the HCNp of *P. vulgaris* was significantly lower than that of the *P. lunatus* genotype 8079 (Fig. 3). In contrast to the distinct differences of HCNp, the concentration of soluble proteins among experimental plants showed no significant differences (according to one-way ANOVA: $F_{2,57} = 1.594$, $P = 0.212$) (Fig. 3).

3.4. Gene expression of cyanase in *T. urticae*

Cyanase was expressed in *T. urticae* larvae, nymphs and female adults (Fig. 4A). The cyanase gene transcript levels were significantly higher in adults when compared to larvae and nymphs. No significant changes in expression levels of cyanase were observed between wild mite populations collected from *P. avium*, *C. sativus* and *S. nigra* when compared to London strain feeding on *P. vulgaris* (Fig. 4B). No significant changes in cyanase transcript levels were observed after 24 h feeding on five new host plants (Fig. 4C). However, after 6 generations, cyanase expression levels significantly increased on *P. lunatus* 8078 and *S. lycopersicum* compared to the ancestral host plant (*P. vulgaris*) (Fig. 4C).

3.5. Recombinant expression and activity assays of *T. urticae* cyanase

His-tagged cyanase of *T. urticae* was successfully recombinantly expressed in *E. coli*. In all of the expression strains tested, the produced protein was mainly present in the insoluble phase, but we could purify mg quantities of soluble proteins from the BL21 (DE3) plys expression strain with Ni²⁺-NTA affinity purification. Western blot analysis with anti-His antibodies revealed the
presence of a single band at 20 kDa (see Fig. S2). Aliquots of dialyzed protein preparations were used in activity assays. The apparent $K_m$ and $V_{\text{max}}$ for both cyanase substrates are listed in Table 2 and compared to reported kinetic parameters of other cyanase enzymes from diverse origin. The $K_m$ (cyanate) value of the $T. urticae$ cyanase was the lowest reported thus far, and points to a high affinity of the enzyme for cyanate. The apparent $V_{\text{max}}$ values were higher than those of the other eukaryotic cyanases, but clearly lower than what has been reported for bacterial enzymes. $T. urticae$ cyanase activity was bicarbonate dependent, similar to what is observed for all characterized cyanases up to date (Elleuche and Poeggeler, 2008; Johnson and Anderson, 1987; Qian et al., 2011). The in vitro activity of $T. urticae$ cyanase was affected by pH and temperature (Fig. 5) and maximum enzyme activity in our assays was observed at around 40 °C and at pH 7.6.

4. Discussion

Lateral gene transfer to or within eukaryotic organisms is considered a rare event, and even less cases have been reported for animals. This might reflect the fact that the animal germ line cells are isolated from somatic cells, which inhibits an efficient transfer of DNA (Andersson, 2005), or that such events are still largely overlooked because of genome sequencing strategies (Hotopp, 2011). The few clear cases of lateral gene transfer in animals have indicated a tendency for the acquisition of genes that allow specialization and adaptation to a new environment (Keeling and Palmer, 2008). For example, it was recently shown how the lateral gene transfer of a bacterial mannanse to the coffee berry borer beetle, Hypothenemus hampei, allows the insect to hydrolyze galactomannan, the main storage polysaccharide in the beetle’s food (Acuna et al., 2012). Other clear examples include the acquisition of fungal carotenoid synthases and cyclases in aphids that allow the synthesis of pigments (Moran and Jarvik, 2010), a transfer that was also recently detected in the spider mite $T. urticae$ (Grbic et al., 2011).

The acquisition of a cyanase gene in $T. urticae$ might be another example of a lateral gene transfer conferring an adaptive benefit in a challenging environment. Animal cyanases were first detected in nematode genomes, and it is established that lateral gene transfer at least partly underlies the evolution toward plant parasitism in nematodes (Haegeman et al., 2011). However, the nematode cyanases have not been functionally expressed and their potential role in plant parasitism was suggested but not investigated. Our phylogenetic analysis of cyanases included nematode proteins and points toward a bacterial origin of nematode cyanases. This confirms an earlier report that nematode cyanases are homologous to bacterial proteins. A potential horizontal transfer from bacteria has been previously suggested, but was not yet supported by a thorough phylogenetic analysis (Guilloton et al., 2002; Haegeman et al., 2011; Opperman et al., 2008). If this is indeed the case, the ancestral bacterial gene gained introns after transfer without interrupting its original open reading frame, which further points toward a potential selective advantage of cyanase activity.

We identified three cyanase sequences within the phytophagous mite family Tetranychidae and show that at least the $T. urticae$ cyanase enzyme was functionally active and metabolizes cyanate with similar kinetics as what has been reported for the plant and fungal eukaryotic enzymes. Phylogenetic analysis suggested a common evolutionary origin for all spider mite cyanases and thus indicated that the lateral gene transfer took place before further speciation within the Tetranychidae. The donor organism could not be resolved in our analysis, probably due to a lack of variation in the short cyanase proteins. We could not detect cyanases in known maternally transmitted endosymbionts of mites, which could have potentially overcome the DNA barrier of reproductive cells.

### Table 2

The kinetic parameters of $T. urticae$ cyanase and earlier reported cyanase proteins. The apparent values for $T. urticae$ cyanase were calculated by double-reciprocal plots (values for $E. coli$ obtained from Anderson (1980); for $S. macrospora$ from Elleuche and Poeggeler (2008) and values for $A. thaliana$ and $O. sativa$ obtained from Qian et al. (2011)).

<table>
<thead>
<tr>
<th>Kinetic parameter</th>
<th>$T. urticae$</th>
<th>$A. thaliana$</th>
<th>$O. sativa$</th>
<th>$S. macrospora$</th>
<th>$E. coli$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyanate $K_m$ (mM)</td>
<td>0.38</td>
<td>0.94</td>
<td>7.38</td>
<td>0.58</td>
<td>0.60</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (mmol/mg*min)</td>
<td>20.040</td>
<td>3.980</td>
<td>2.080</td>
<td>3.163</td>
<td>&gt;323</td>
</tr>
<tr>
<td>Bicarbonate $K_m$ (mM)</td>
<td>2.56</td>
<td>0.79</td>
<td>0.63</td>
<td>3.163</td>
<td>0.1</td>
</tr>
<tr>
<td>$V_{\text{max}}$ (mmol/mg*min)</td>
<td>19.874</td>
<td>3.820</td>
<td>0.982</td>
<td>3.163</td>
<td>&gt;323</td>
</tr>
</tbody>
</table>
particular notice, our analysis of genome and transcriptome data did not detect cyanase genes in non-plant feeding Acari. Amongst mites, by far the most diverse lineages that have adapted to phytophagy are the Eriophyoidea and Tetranychidae (including spider mites) (Lindquist, 1998), and although speculative, a horizontal gene transfer might have played a role in the transition of the ancestral predaceous lifestyle to phytophagy in these lineages (Walter and Proctor, 1999), similar to what has been suggested for cyanases in plant parasitic nematodes (Haegeman et al., 2011).

If the ability to metabolize cyanate confers a direct evolutionary advantage for spider mites, it could be expected that cyanase is present in their environment. However, cyanase is not a common plant metabolite and amounts present in plant cell juice probably do not convey a selection pressure by acute toxicity alone. However, cyanate can be formed by oxidation of HCN, a common plant metabolite, and amounts present in plant cell juice probably do not convey a selection pressure by acute toxicity alone. However, cyanate can be formed by oxidation of HCN, a common plant defense molecule in cyanogenic plants (Poulton, 1990). Oxidation can occur either by the cyanide mono-oxgenase as has been reported for micro-organisms, by spontaneous photo-oxidation, or indirectly via thiocyanate as an intermediary (Ebbs, 2004; Gupta et al., 2010). Hence, as a first hypothesis in search of a functional role for T. urticae cyanase, we tested whether cyanase expression levels change when mites were transferred from acyanogenic P. vulgaris plants to P. lunatus accessions with different cyanogenic potential. It has been previously shown that spider mite herbivory induces β-glucosidase activity in P. lunatus and as a consequence the release of HCN (Ballhorn et al., 2006). In this controlled experiment of host plant transfer, we observed a significant increase of cyanase mRNA levels after feeding for 6 generations on a cyanogenic P. lunatus genotype (Fig. 4), suggesting that cyanase activity could act as a potential mediator of cyanide detoxification. In contrast, cyanase expression levels in the two wild T. urticae populations collected on known cyanogenic plants (elderberry and cherry) (Atkinson and Atkinson, 2002; Santamour, 1998) did not significantly differ when compared to expression levels of the reference strain feeding on acyanogenic P. vulgaris.

Although cyanogenesis is not a reported defense mechanism in S. lycopersicum (Jones, 1998), the recent release of the tomato genome sequence does reveal the presence of a CYP79 which is indicative of the biosynthesis of either cyanogenic glucosides or glucosinolates (Bak et al., 2006; Sato et al., 2012). Still, other plant related stress-factors, such as the nutritional value of plants, might also affect cyanase expression. A correlation of cyanase gene expression and environmental stress has previously been documented in fungi and bacteria (Elleuche and Poeggeler, 2008; Guilloton and Karst, 1987b; Kunz and Nagappan, 1989) and also in plants cyanase expression can be induced by salt stress (Qian et al., 2011).

Alternatively, cyanate is also formed by spontaneous dissociation of carbamoyl phosphate in vivo (Guilloton and Karst, 1987a; Lawrie, 1979), which is the main substrate leading to the synthesis of pyrimidines and the amino acid arginine in both eukaryotes and prokaryotes (Holden et al., 1999; Pierard et al., 1976). Carbamoyl phosphate is synthesized by the universal carbamoyl phosphate synthetase (after Elleuche and Poeggeler (2008) and this study): (1) carbamoyl phosphate synthetase (CPS) catalyzes the conversion of glutamine, bicarbonate (gray shaded) and ATP into carbamoylphosphate (Holden et al., 1999), (2) decomposition of carbamoylphosphate into cyanate (Guilloton and Karst, 1987a; Lawrie, 1979), (3) cyanase catalyzes the decomposition of cyanate into ammonia and carbon dioxide (Johnson and Anderson, 1987); cyanase and CPS compete for bicarbonate (gray shaded), which is a substrate in both reactions, (4) oxidation of HCN (e.g. released by cyanogenic glucosides of plants) into cyanate could occur either by a cyanide mono-oxygenase or photo-oxidation (Ebbs, 2004; Gupta et al., 2010; Malhotra et al., 2005), (5) reversible inhibition of CPS by cyanate (Guilloton and Karst, 1987a) and (6) presence of high levels of arginine leads to down-regulation of cyanase in the fungus S. macrospora (Elleuche and Poeggeler, 2008).

![Fig. 5. Influence of pH (left) and temperature (right) on the in vitro activity of T. urticae cyanase using standard assay conditions.](image-url)

![Fig. 6. An overview of possible interactions between cyanase and diverse pathways related to carbamoylphosphate synthase (after Elleuche and Poeggeler (2008) and this study): (1) carbamoylphosphate synthetase (CPS) catalyzes the conversion of glutamine, bicarbonate (gray shaded) and ATP into carbamoylphosphate (Holden et al., 1999), (2) decomposition of carbamoylphosphate into cyanate (Guilloton and Karst, 1987a; Lawrie, 1979), (3) cyanase catalyzes the decomposition of cyanate into ammonia and carbon dioxide (Johnson and Anderson, 1987); cyanase and CPS compete for bicarbonate (gray shaded), which is a substrate in both reactions, (4) oxidation of HCN (e.g. released by cyanogenic glucosides of plants) into cyanate could occur either by a cyanide mono-oxidase or photo-oxidation (Ebbs, 2004; Gupta et al., 2010; Malhotra et al., 2005), (5) reversible inhibition of CPS by cyanate (Guilloton and Karst, 1987a) and (6) presence of high levels of arginine leads to down-regulation of cyanase in the fungus S. macrospora (Elleuche and Poeggeler, 2008).](image-url)
synthetase enzyme (CPS) (Lawson et al., 1996) and this enzyme is reversibly inhibited by cyanate (Guilloton and Karst, 1987a). Due to this by-product inhibition, it was previously suggested that cyanase could act as a regulatory enzyme in these synthesis pathways (Guilloton et al., 2002). This is further supported by the fact that cyanase is transcriptionally regulated by arginine in the fungus S. macrospora (Elleuche and Poeggeler, 2008). In addition, CPS and cyanase can compete for bicarbonate, which is a substrate in both pathways depicted in Fig. 6. In conclusion, cyanase activity might be regulated by mechanisms such as inhibition by cyanate and/or competition for bicarbonate.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2012.08.002.

References


