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How arthropod herbivores develop
a taste for poison

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Cyanide, detoxification, lateral gene transfer, phytophagy

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Adaptation of animal herbivores to novel plant hosts is severely limited by the diverse set of toxic chemicals that plants produce to defend themselves. Arthropod herbivores nonetheless display a high adaptive potential towards novel hosts and, as a result, can have a very dynamic host plant range. The molecular mechanisms that underlie arthropod adaptation to anti-herbivore plant defenses are largely unknown. In my thesis, we aimed to explore and characterize these unknown molecular mechanisms using the two-spotted spider mite *Tetranychus urticae* as a model system with available genomic resources. Experimental evolution of *T. urticae* populations showed that, upon host plant adaptation, mites exhibited an increased transcriptional plasticity of detoxification genes, which likely leads to a higher detoxification potential. Within the gene set that responded to host plant adaptation, a number of genes were discovered whose presence in the mite genome can best be explained by horizontal gene transfer from microorganisms. *In silico* characterization and functional expression indicated that the horizontally transferred genes in *T. urticae* and related spider mite species underlie their ability to neutralize plant-produced toxins. Finally, we tested at the molecular level whether host plant adaptation can indeed facilitate the development of pesticide resistance, a hypothesis that has been and remains a topic of some debate among entomologists.

Introduction

Plants synthesize defensive compounds, or allelochemicals, to counter herbivore attacks. These allelochemicals repulse and poison herbivores, and interfere with the assimilation of nutritive plant compounds within the herbivore's digestive tract. As a response, herbivores have evolved various strategies to neutralize plant allelochemicals. Many of these adaptation processes occur within the physiology of the herbivore. Here, certain enzymes, typically encoded by ubiquitous multi-gene families, prevent that ingested allelochemicals become toxic. These detoxifying enzymes constitute the xenobiotic metabolism of an organism and are traditionally divided into three distinct phases: (i) metabolism, where the structure of the toxin is enzymatically altered, (ii) conjugation of the toxin with another compound, and (iii) translocation of the toxin over membranes (see also the schematic overview in figure 1).

Most arthropod herbivores only feed on a specific or a few closely related host plants and have specialized in disarming the toxins produced by plants within their narrow host range. For instance, the cinnabar moth (*Tyria jacobaeae* (Linnaeus)), which flutters about in Belgian and Dutch coastal areas, only feeds on ragwort plants. These plants defend themselves against herbivore feeding by producing toxic pyrrolizidine alkaloids in their leaves. Not only can the specialized larvae of *T. jacobaeae* ingest these alkaloids without harm, they even store and accumulate the toxins in a process called sequestration. By sequestering plant toxins, *T. jacobaeae* becomes less attractive

for insectivores. Biochemical and genetic experiments identified the detoxification enzyme as a special flavin-dependent monooxygenase, present in the haemolymph of this black-and-red arctiid moth specialist (Naumann *et al.* 2002). However, in most systems, the exact enzyme(s) that enables arthropod herbivores to handle plant defenses remains enigmatic. A famous case that is puzzling entomologists for over a century is how certain arthropod groups overcome plant cyanogenesis, an ancient and widespread anti-herbivore defense mechanism (Zagrobelny *et al.* 2004). Cyanogenic plants deter herbivores from feeding by releasing toxic cyanide in their disrupted tissues. As cyanide is an extremely potent toxin (lethal to humans at a mere dosage of approximately 2 mg/kg body weight), cyanogenesis is a highly effective defense mechanism. Nonetheless, certain lepidopterans and plant feeding spider mites are able to detoxify plant produced cyanide and thrive on cyanogenic plants.

The two-spotted spider mite *Tetranychus urticae* (Koch) (*bonenspintmijt* in Dutch) is a classic example of an arthropod generalist that successfully feeds on over 1,000 plant species across more than 140 plant families (figure 2) (Migeon *et al.* 2017). Generalists such as *T. urticae* seem to rely on an enhanced xenobiotic metabolism to detoxify the numerous different allelochemicals present in their diverse plant diet (Krieger *et al.* 1971). In his seminal paper of 1961, Gordon argued that in the eyes of an arthropod herbivore, a man-made pesticide is similar to a newly encountered plant allelochemical and that arthropods

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Tijdens de 28e Nederlandse Entomologendag (Ede, 16 december 2016) is de negende NEV-Dissertatieprijs uitgereikt aan Dr. Nicky Wybouw, voor zijn proefschrift 'The role of horizontally transferred genes in the xenobiotic adaptations of the spider mite *Tetranychus urticae*', op 9 oktober 2015 verdedigd aan de Universiteit van Amsterdam. De prijs bestaat uit een geldbedrag plus een oorkonde en wordt jaarlijks toegekend voor het beste proefschrift op het gebied van de entomologie, verdedigd aan een Nederlandse universiteit in het voorgaande academische jaar (1 september – 31 augustus). Het juryrapport roemt de indrukwekkende combinatie van experimenten aan een vernieuwend en belangwekkend onderwerp: de rol van horizontale genoverdracht voor de evolutie van herbivorie in mijten en insecten. De experimentele hoofdstukken zijn wetenschappelijk sterk. Naast de fundamentele insteek heeft het proefschrift ook een duidelijke link met de toepassing (o.a. pesticide resistentie). Het proefschrift is goed geschreven met veel

diepgang in de inleiding en discussie, en daarin ook een sterke vooruitblik naar hoe vervolgonderzoek breder kan worden getrokken naar andere insectentaxa.

During the 28th Annual Dutch Entomologists Meeting (Ede, 16 December 2016), the ninth Netherlands Entomological Society (NEV) Dissertation Award was presented to Dr. Nicky Wybouw, for his thesis 'The role of horizontally transferred genes in the xenobiotic adaptations of the spider mite *Tetranychus urticae*', defended on 9 October 2015 at the University of Amsterdam. This prize comprises a sum of money and a certificate of appreciation, and is awarded for the best doctoral thesis in the field of entomology, defended at a Dutch university in the preceding academic year (1 September – 31 August). The committee valued the impressive combination of experiments on an original and important topic: the role of horizontal gene transfer in the evolution of herbivory in mites and insects. The experimental



chapters are of high scientific quality. In addition to the fundamental focus of the dissertation, there is also a direct link with applied research (e.g. pesticide resistance). The thesis is well written with a good level of depth in the introduction and discussion, including a strong perspective on how future research could be extended to other insect taxa.

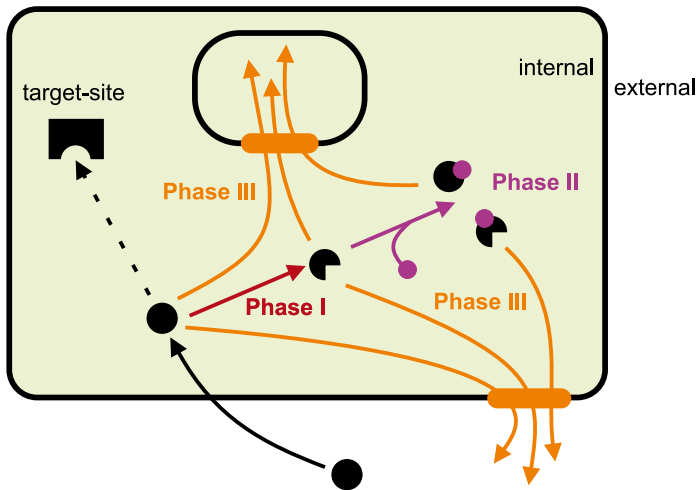
co-opt their pre-evolved detoxification pathways for plant allelochemicals to detoxify synthetically produced pesticides. He also hypothesized that the augmented detoxification capacity of generalists strongly facilitates the evolution of pesticide resistance (Gordon 1961). Interestingly, the extremely polyphagous *T. urticae* quickly develops pesticide resistance and multi-resistant mite populations often emerge in greenhouse and field crops (Van Leeuwen *et al.* 2010). However, the interplay between host plant adaptation and pesticide resistance remains a heavily debated topic within the scientific community. Although some studies provide support for the pre-adaptation hypothesis, others argue that taxonomy and feeding guild influence their conclusions.

In 2011, an international research consortium, to which my Ph.D. supervisor Thomas Van Leeuwen belongs, sequenced and analyzed the genome of *T. urticae* using Sanger technology (Grbic *et al.* 2011). To their surprise, they discovered that this big pest possesses a tiny genome, spanning only 90 Mb (or megabasepairs). In comparison, the genome size of the fruit fly *Drosophila melanogaster* is approximately 180 Mb (Adams *et al.* 2000). Even more surprising, multi-gene families that are known to code for detoxifying enzymes possess record numbers of gene copies within the small mite genome (Dermauw *et al.* 2014, Grbic *et al.* 2011). Taking advantage of this genomic resource, our team designed a molecular tool to analyze and compare the complete transcriptomes of different *T. urticae* populations.

Host plant adaptation of a generalist herbivore

In a first study, we used this transcriptomic tool to shed light on the enigmatic molecular mechanisms that underlie the high

potential for host plant adaptation in generalist herbivores (Wybouw *et al.* 2015). Here, we focused on the adaptation processes of *T. urticae* to tomato. Tomato plants produce a wide spectrum of defensive compounds that are known to be challenging for certain spider mite populations. In our laboratory, such a susceptible mite population was transferred from its ancestral bean host to tomato where three selection lines were allowed to propagate. Performance tests revealed that the reproductive output of these selection lines already quadrupled after 30 generations, compared to non-adapted mites. This significant increase in reproductive output was stable, regardless whether the selection lines were fed with bean or tomato. The tomato selection lines were therefore shown to be genetically adapted to the new host. Moreover, our tests also showed that tomato adaptation increased the host plant range of the selection lines. In other words, after tomato adaptation, the selection lines did not experience a detectable fitness cost on other hosts. These results confirmed previous experimental evolutionary studies where no negative genetic correlations were found in the performance of generalists on host plants. Subsequently, we sampled the transcriptomes from non-adapted and tomato-adapted mite populations under various plant feeding regimes. Remarkably, large transcriptomic rearrangements were observed across both feeding regime and adaptation status (a total of 1,275 differentially expressed genes). More detailed transcriptomic analysis uncovered two main mechanisms associated with tomato adaptation. First, tomato adaptation resulted in a set of mite genes that were constitutively (i.e. independent of the feeding regime) down-regulated in the adapted mites compared to the non-adapted mites. Several of these constitutively down-regulated genes



1. Enzymatic detoxification in herbivores. The xenobiotic metabolism handles an ingested allelochemical in three separate phases: metabolism (I), conjugation (II) and translocation (III). Enzymes of different phases often work in unison to completely eliminate the poisonous effects of an allelochemical. Through the enzymatic actions within phase I and II, lipophilic xenobiotics become more water soluble and easier to excrete during phase III.

1. Enzymatische ontgiftiging in herbivoren. Enzymen worden in drie fasen geclassificeerd, afhankelijk van hun interactie met het opgenomen toxine. Deze fasen zijn: metabolisatie (I), conjugatie (II) en transmembranaire verplaatsing (III). Vaak moet een toxine door alle fasen gaan voordat het compleet geneutraliseerd is.

belong to multi-gene families that code for regulatory enzymes. Certain classes of regulatory enzymes dictate the transcription levels of other genes by interacting with the genome. In our experimental evolutionary set-up, the constitutive down-regulation of these regulatory genes might thus be causing the observed massive transcriptomic rearrangements. Second, tomato-adapted mites mounted a stronger transcriptional response when re-exposed to tomato for 24 hours, compared to non-adapted mites. Bio-informatic characterization revealed that, within this short-term transcriptomic response, adapted mites recruited more components of their detoxification repertoire. Specifically, genes coding for enzymes

that metabolize and transport allelochemicals showed enrichment. Using *T. urticae* as a model, this study indicated that a constitutive down-regulation of regulatory genes and an increased transcriptional plasticity of detoxification genes play a central role in host plant adaptation of polyphagous herbivores.

A link with pesticide resistance

In a series of experiments spearheaded by Wannes Dermauw, we tested the pre-adaptation hypothesis at the molecular level (Dermauw *et al.* 2013). We included two mite populations in our transcriptomic analyses that were collected from heavily sprayed field crops and are known to be strongly resistant to various pesticides. Compared to a pesticide susceptible population, the two multi-resistant strains differentially expressed a wide range of genes that code for enzymes of the xenobiotic metabolism. Interestingly, the transcriptional signatures of these multi-resistant strains closely resembled those of tomato-adapted mites. Indeed, genes coding for detoxifying enzymes showed a strong correlation in their transcriptional patterns. Toxicity bioassays also showed that the tomato-adapted mites had an increased tolerance to three unrelated pesticides, compared to non-adapted mites. Our results therefore supported an evolutionary model wherein generalist arthropods have developed the ability to mount a broad transcriptomic response upon host plant adaptation and that this transcriptomic plasticity predisposes the evolution of pesticide resistance in generalists.

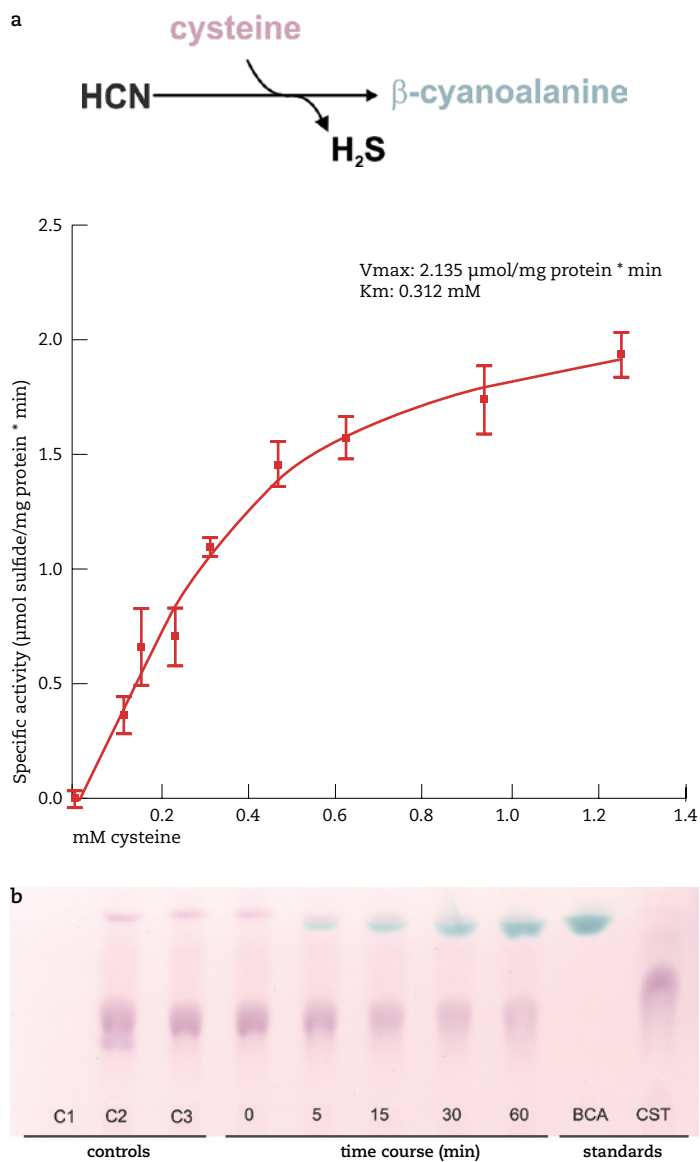
A newly discovered route of detoxification?

A *T. urticae* gene family coding for intradiol ring-cleavage dioxygenases showed significant differential expression upon tomato adaptation and pesticide resistance (Dermauw *et al.* 2013, Wybouw *et al.* 2015). This family of dioxygenases does not normally occur within animals and is restricted to bacteria and fungi where it plays an important role in the metabolism of compounds with a recalcitrant aromatic ring. Intradiol ring-cleavage dioxygenases have the unique ability to cleave stable aromatic rings between adjacent hydroxyl groups. PCR amplification on the genomic DNA of related spider mite species



2. An adult female *Tetranychus urticae* with two eggs on a bean leaf. Photo: Jan van Arkel

2. Een volwassen *Tetranychus urticae*-vrouwtje met twee eieren op een bonenblad.



3. (a) The β -cyanoalanine synthase reaction with accompanying kinetic plot of the recombinant *T. urticae* enzyme. (b) Thin Layer Chromatographic visualization of the formation and accumulation of β -cyanoalanine by recombinant *T. urticae* β -cyanoalanine synthase. Controls were C1: no cysteine, C2: no cyanide and C3: no enzyme. Time course went from 0 to 60 min. Standards were BCA: β -cyanoalanine and CST: cysteine.

3. (a) Cyanide-ontgiftigingsreactie, gekatalyseerd door β -cyanoalanine synthase, en de enzymkinetiek van het recombinante mijtenenzym. (b) Visualisatie van het β -cyanoalanine-product na incubatie met het recombinante mijtenenzym op een TLC-plaat. Controles zijn: C1: geen cysteine, C2: geen cyanide en C3: geen enzym. Reacties werden stopgezet op verschillende tijdstippen (0 tot 60 min). Standaarden zijn: BCA: β -cyanoalanine en CST: cysteine.

showed that *Tetranychus* species possess multiple intradiol ring-cleavage dioxygenase gene copies. Phylogenetic analysis strongly pointed towards an evolutionary scenario wherein an intradiol dioxygenase gene was transferred once from a fungal donor species into an ancestral tetranychid species through the process of horizontal gene transfer (HGT) and subsequently duplicated, hereby forming multi-gene families in descendent mite species. This was highly surprising as HGT, defined as the process whereby genetic information is asexually transferred between reproductively isolated species, has traditionally been considered to only occur among bacterial and simple, unicellular eukaryotic species. In these microbes, HGT is a strong evolutionary force, driving adaptation to biotic and abiotic factors.

Interestingly, many plant allelochemicals, including those produced by tomato, as well as pesticides possess various aromatic structures to ensure stability. Plant pathogenic fungi secrete intradiol ring-cleavage dioxygenase enzymes to metabolize aromatic plant allelochemicals in *plantae* (Roopesh et al. 2010). The lateral acquisition of these enzymes might thus have provided plant feeding spider mites with a completely new detoxification route. We are presently functionally characterizing these novel genes and further investigating their potential role in the xenobiotic metabolism of mites.

Cyanide detoxification in arthropods

In a subsequent study using *T. urticae* as a model, we focused on unraveling the molecular basis of arthropod adaptation to plant cyanogenesis (Wybouw et al. 2012, Wybouw et al. 2014). A *T. urticae* population was allowed to adapt to a cultivar of cyanogenic lima bean, which releases high levels of cyanide upon herbivore attack, as shown by our biochemical tests. In sharp contrast to tomato adaptation, transcriptomic analysis revealed that mites over-expressed a very limited set of genes upon lima bean adaptation. Interestingly, lima bean-adapted mites over-expressed two genes that were laterally acquired from microbial donor species, like intradiol ring-cleavage dioxygenases. Both horizontally transferred genes were recombinantly expressed in *E. coli* and functionally characterized to investigate whether the mite enzymes catalyze similar reactions as their microbial counterparts. Enzyme assays showed that one of the laterally transferred genes codes for an active cyanase enzyme that converts cyanate, a bacterial and spontaneous decomposition product of cyanide, and bicarbonate into ammonia and carbon dioxide. Since cyanase metabolizes a cyanide-derived compound, we were encouraged to further investigate whether spider mites overcome plant cyanogenesis by expressing cyanase. However, our results did not support this hypothesis but favored another, more basic, role for cyanase in mite physiology (Wybouw et al. 2012). First, transcriptional induction was also observed when *T. urticae* mites were placed on other, non-cyanogenic plants and, second, non-phytophagous mites with highly variable lifestyles also possess horizontally transferred cyanase genes (Schlachter et al. 2017, Wybouw et al. 2012).

Functional expression of the second laterally transferred gene showed that, like its close bacterial homologues, it codes for an active β -cyanoalanine synthase that directly metabolizes toxic cyanide. Specifically, the β -cyanoalanine synthase enzyme converts cyanide and the amino acid cysteine into the amino acid derivative β -cyanoalanine and hydrogen sulfide. Multiple chromatographic analyses (TLC and HPLC) confirmed the formation of β -cyanoalanine when incubated with the mite enzyme and cyanide (figure 3). Enzyme kinetics also showed that the horizontally transferred β -cyanoalanine synthase catalyzes this detoxification reaction highly efficiently. To unambiguously prove that the bacterial β -cyanoalanine synthase gene was physically incorporated into the *T. urticae* genome, we mined Sanger- and Illumina-sequenced genome assemblies of several mite populations and showed a continuous read coverage in large scaffolds that hold normal, eukaryotic neighbouring genes. When we subsequently screened other arthropod genomes, we saw that only lepidopteran genomes also harbor a microbial β -cyanoalanine synthase gene (Wybouw et al. 2014). Later studies have shown that the lepidopteran enzymes also remained active after the HGT event and are able to detoxify cyanide (van Ohlen et al. 2016). Interestingly, some lepidopteran species that have specialized to feeding on cyanogenic plants duplicated their gene copy and now hold a small family of β -cyanoalanine synthase genes in their genome.

Horizontal gene transfer contributes to the evolution of arthropod herbivory

The above study showed that a gene horizontally transferred from bacteria was co-opted by two lineages of arthropods to detoxify plant-produced cyanide and is a clear example of how HGT can be a driving force in animal evolution. These findings corroborate other experimental evidence that strongly indicates that genomes of a wide set of plant feeding arthropod species harbor a number of horizontally transferred genes. Moreover, as reviewed in Wybouw *et al.* (2016), these novel genes underpin many adaptations to phytophagy which are not restricted to detoxification but also include an efficient assimilation of plant metabolites. Horizontally acquired genes and the traits they encode typically functionally diversify within arthropod recipients, enabling the colonization of more host plant species and tissues. By uncovering its prominent role in the adaptations of arthropods to exploit plants, we show that HGT can be of functional importance in metazoan evolution (Wybouw *et al.* 2016).

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I would like to take this opportunity to thank Thomas Van Leeuwen, my Ph.D. supervisor, for all his advice and help throughout my Ph.D. thesis. I am also indebted to Wannes Dermauw for leading the experiments on the link between host plant adaptation and pesticide resistance. I would also like to thank Sabina Bajda for all her love and never-ending support and the Dutch Entomology Society for giving me the platform to share my and my colleagues' research. Our work on intradiol ring-cleavage dioxygenases is supported by an FWO project (G009312N to Thomas Van Leeuwen) and a Marie Skłodowska-Curie Action Individual fellowship (658795-DOGMITE to Nicky Wybouw).

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Samenvatting

Waarom de bonenspintmijt een boontje heeft voor zoveel giftige planten

Planten produceren een breed spectrum aan toxines om zich tegen vraatzuchtige insecten en mijten te beschermen. Deze geleedpotige herbivoren passen zich echter relatief snel aan nieuwe waardplanten en hun gifarsenaal aan. Het doel van mijn proefschrift was om de moleculaire mechanismen die deze adaptatieprocessen aansturen bloot te leggen. We kozen als modelsysteem de extreem polyfage bonenspintmijt, *Tetranychus urticae*. Er werden selectielijnen gecreëerd op twee giftige planten: tomaat en limaboon. De transcriptieniveaus van alle genen in het mijtengenoom werden vervolgens bepaald en vergeleken met deze in de moederlijnen. Er konden twee transcriptiepatronen geïdentificeerd worden die gepaard gingen met waardplantadaptatie: een stabiele aanpassing en een plastische verandering waarbij de transcriptie verhoogt na blootstelling aan de toxische waardplant. De genen die coderen voor enzymen die toxines ontgiften volgden hoofdzakelijk het tweede patroon. De transcriptionele profielen werden vervolgens vergeleken met deze van mijtenpopulaties die resistent zijn tegen allerlei pesticiden. Zo testten we de pre-adaptatiehypothese die stelt dat waardplantadaptatie pesticideresistentie kan aansturen. In de set van mijtengenomen met interessante transcriptiepatronen vonden we een aantal die horizontaal verworven zijn van microbiële donororganismen, en dus uniek zijn in de dierenwereld. Functionele expressie toonde aan dat deze unieke genen nog steeds coderen voor actieve enzymen en zelfs verantwoordelijk zijn voor het ontgiften van het toxine dat geproduceerd wordt door limaboon.



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