Molecular Mechanism of the Two-Component Suicidal Weapon of *Neocapritermes taracua* Old Workers

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Abstract

In termites, as in many social insects, some individuals specialize in colony defense, developing diverse weaponry. As workers of the termite *Neocapritermes taracua* (Termitidae: Termitinae) age, their efficiency to perform general tasks decreases, while they accumulate defensive secretions and increase their readiness to fight. This defensive mechanism involves self-sacrifice through body rupture during which an enzyme, stored as blue crystals in dorsal pouches, converts precursors produced by the labial glands into highly toxic compounds. Here, we identify both components of this activated defense system and describe the molecular basis responsible for the toxicity of *N. taracua* worker autothysis. The blue crystals are formed almost exclusively by a specific protein named BP76. By matching *N. taracua* transcriptome databases with amino acid sequences, we identified BP76 to be a laccase. Following autothysis, the series of hydroquinone precursors produced by labial glands get mixed with BP76, resulting in the conversion of relatively harmless hydroquinones into toxic benzoquinone analogues. *Neocapritermes taracua* workers therefore rely on a two-component activated defense system, consisting of two separately stored secretions that can react only after suicidal body rupture, which produces a sticky and toxic cocktail harmful to opponents.

Key words: **altruism, colony defense, termite, Isoptera, laccase.**

Article

Introduction

Members of social insect colonies are partitioned into castes that specialize in colony tasks, such as reproduction, foraging for food, feeding dependent individuals, or defending the colony (Wilson 1971). This task partitioning is often associated with multiple morphological adaptations distributed among castes. Among the manifold morphological adaptations, the most extreme ones are probably those connected to colony defense, such as the enlarged mandibles and defensive glands producing a wide array of defensive compounds (Prestwich 1984; Šobotník, Jirošová, et al. 2010).

Social insects colonies can count up to millions of individuals, attracting many predators, and being subjected to intense competition from neighboring colonies (Lepage and Darlington 2000), making colony defense essential. In the most complex insect societies, defense is entrusted to sterile individuals, which often constitute a specialized soldier caste. Social Hymenoptera defenders have developed sophisticated strategies, which in extreme cases involve self-destructive processes, such as sting autotomy in some bees, wasps, and ants (Hermann 1971), or autothysis, that is, body rupture to release defensive substances from specialized glands, in some *Camponotus* (*Colobopsis*) ant species (Maschwitz and Maschwitz 1974). Termite defense strategies are comparably diverse, and although the weaponry is particularly developed in soldiers, workers may actively take part in colony defense (Thorne 1982), especially in soldierless species (Sands 1982). Suicidal defense is common in soldiers of many species that keep their mandibles locked in the opponent's wound (Deligne et al. 1981; Prestwich 1984). Like *Camponotus* ants, some termite soldiers or workers can sacrifice themselves, and perform autothysis to release defensive secretions by body rupture, or dehiscence, during which the inner organs burst out of the abdomen. While dehiscence mostly occurs in workers of soldierless termites (Sands 1982), autothysis evolved independently in soldiers of Serritermitidae (Costa-Leonardo

and Kitayama 1991; Šobotník, Bourguignon, et al. 2010), *Globitermes* (Bordereau et al. 1997), *Dentispicotermes* (Šobotník, Jirošová, et al. 2010), and *Apilitermes* (Deligne and De Coninck 2006), and in workers of *Ruptitermes* (Costa-Leonardo 2004) and *N. taracua* (all Termitidae) (Šobotník et al. 2012, 2014).

Termite defensive secretions include a rich set of compounds, terpenoids produced by the frontal gland or acetate-derived quinones produced by labial glands being the most common (Prestwich 1984; Šobotník, Jirošová, et al. 2010). Although these compounds are in general synthesized de novo (Prestwich et al. 1981), the biosynthetic enzymes are largely unknown, except for geranylgeranyl diphosphate synthase involved in diterpene synthesis in *Reticulitermes* and *Nasutitermes* (Hojo et al. 2007, 2011). Although proteins are also frequently used as toxins or venoms in animals (Bettini 1978; Casewell et al. 2013), and despite the presence of unidentified proteins responsible for stiffening after air exposure in glandular secretions of *Mastotermes* and *Odontotermes* (Moore 1968; Wood et al. 1975), no such role has been reported in termites so far.

Neocapritermes taracua (Termitidae: Termitinae) nests and foraging areas are defended by rare soldiers, and by workers that use controlled autothysis, in which the labial gland secretion is converted into toxic compounds by a highly abundant protein stored as blue crystals in exterior pouches (Šobotník et al. 2012). The "explosive backpacks" are only ready-to-use in older workers (hereafter called blue workers) in which the bursting liquid is sticky and toxic to termite opponents. Young workers (hereafter called white workers) also have the potential to commit autothysis but do so less readily and release negligible amounts of toxic secretions (Šobotník et al. 2012, 2014).

Here, we describe the molecular mechanism underlying the toxicity of the bursting liquid of old *N. taracua* workers (Šobotník et al. 2012, 2014), in which two sets of glands are involved. The labial glands secrete the precursors that, after body rupture, react with a blue copper protein produced by specialized crystal glands. We combined tissue-specific transcriptome sequencing of both white and blue *N. taracua* workers with proteomics to identify a candidate laccase named BP76. Next, we investigated the expression pattern of BP76 and five additional laccases identified in *N. taracua* across different tissues of white and blue workers. Finally, we identified the compounds produced by the labial glands of workers and determined the enzymatic activity of BP76 that converts these compounds into harmful analogues after autothysis.

Results

Identification of BP76

Blue crystals isolated from dorsal pouches of blue workers were solubilized and their content separated with denatured gel electrophoresis (sodium dodecyl sulfate–polyacrylamide gel electrophoresis [SDS–PAGE]; see supplementary fig. S1, Supplementary Material online). Coomassie staining of the gel revealed a single dominant band, named BP76 protein that forms most of the blue crystals mass (Šobotník et al. 2012). To enable unambiguous identification of the candidate protein(s) and subsequently analyze tissue-specific gene expression levels, we performed NextGen sequencing (RNAseq) with RNA isolated from 1) body without gut, labial gland, and crystal gland, 2) labial glands, and 3) crystal glands of white and blue *N. taracua* workers. We combined the tissue-specific data sets to build the de novo transcriptome assembly (TA) that we used to identify the cDNA sequence of BP76 as well as related proteins. Three different proteomic methods were employed to identify BP76, which included data-independent (Edman sequencing) and data-dependent mass spectrometry (MS-E and MS/MS) methods. Combining the three different proteomics approaches resulted in the identification of peptides that covered about half the predicted BP76 protein sequence (see supplementary fig. S2, Supplementary Material online). We subsequently carried out a local BLAST search against the *N. taracua* TA and identified the complete cDNA sequence of BP76 (GenBank accession number: KT945242). The best NCBI BLAST hit showed that BP76 is 69% similar to a protein of the termite *Zootermopsis nevadensis* assigned to L-ascorbate oxidase (GenBank accession number: KDR11748.1). The next most similar proteins were laccases of *Reticulitermes flavipes* (GenBank accession number: ACX54558.1–ACX54565.1) and *Z. nevadensis* (GenBank accession number: KDR11747.1) that shared slightly more than 50% similarity with BP76. Further searches against the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) identified three cupredoxin domains characteristic of multicopper oxidases. These results confirm that BP76 is an oxidoreductase and a multicopper protein that we assigned to insect laccases (see Enzymatic Essays).

Phylogenetic Analysis of Laccases

The TA-contig sequences of the de novo reference assembly were translated using BLASTx, searched against the NCBI nr database and functionally annotated by gene ontology and enzyme commission number analysis. Subsequent screening for cDNAs encoding laccases resulted in the identification of a total of six candidate genes, of which we discarded two for a phylogenetic analysis, as the identified cDNAs encoded for less than half of the complete proteins. Based on an amino acid alignment combining laccases from different insect orders (see supplementary fig. S3, Supplementary Material online), we then compared the relative positions of the four *N. taracua* laccases, using Maximum Likelihood (ML) inferred phylogenetic analyses. These analyses showed a disjoint distribution of insect laccases, resulting in at least four separate clades. Each of the four *N. taracua* laccases clustered with a different clade, with all of the critical nodes well supported (node support values of 80% or above). *Neocapritermes taracua* candidate laccase BP76 is placed within a group of termite sequences, including *Zootermopsis* and *Reticulitermes* laccases but excluding other insect laccases (fig. 1).

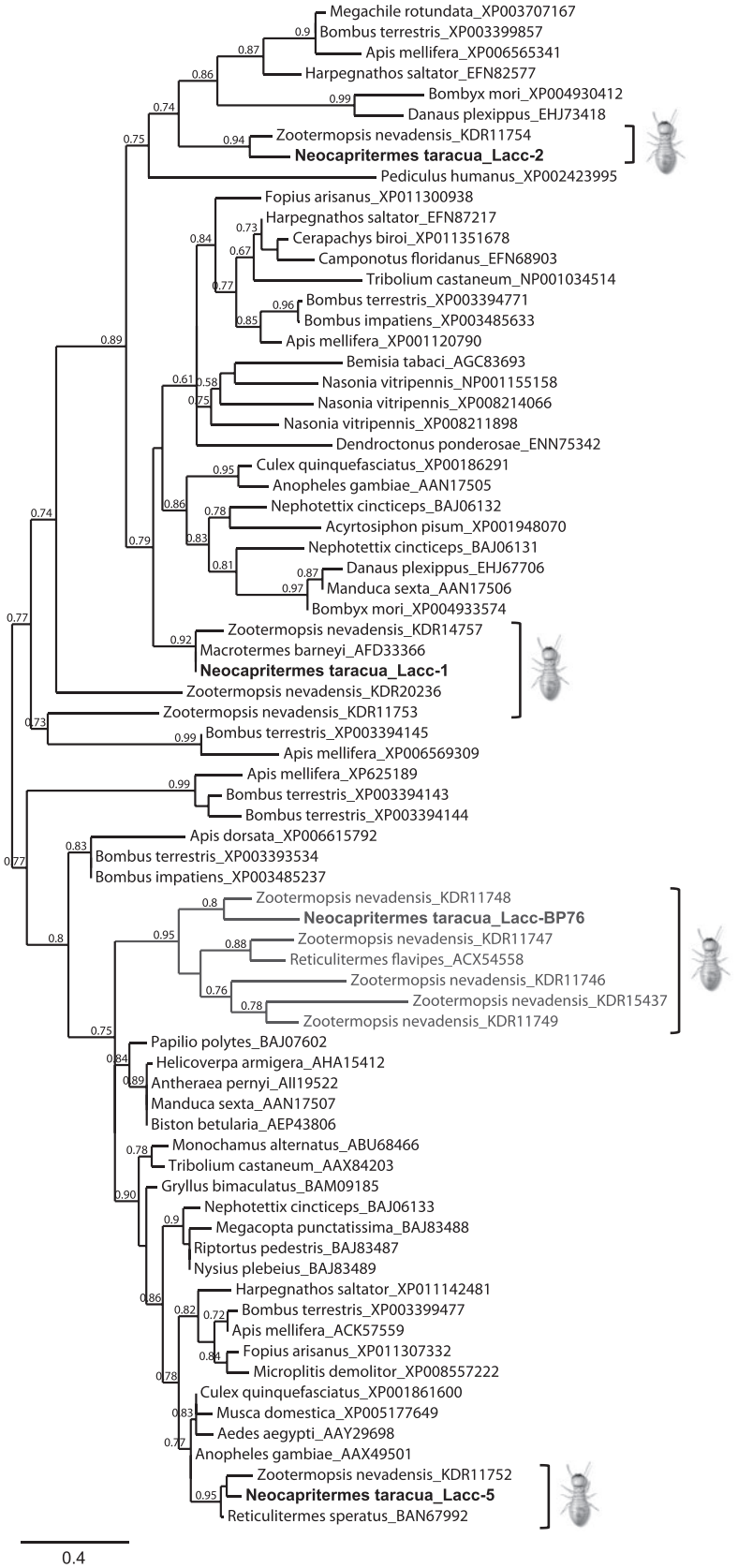

Fig. 1. Phylogenetic tree of insect laccases reconstructed with ML method. Nodes and tips in blue are the termite cluster to which the laccase BP76 belongs; other *Neocapritermes taracua* laccases marked in bold. Species names are followed by NCBI GenBank accession numbers. Branch lengths are proportional to evolutionary distance according to the provided scale. Numbers along branches indicate bootstrap percentage support. Bootstrap support values less than 50% are not shown.

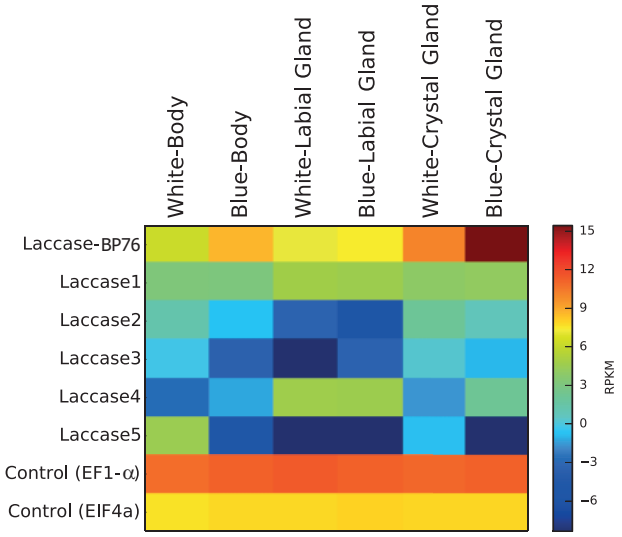

Fig. 2. Heat map showing relative expression levels of the laccase BP76 and five additional laccases in different *Neocapritermes taracua* tissues. Abbreviations for individual laccases as well as two control genes are shown on the left whereas tissues are shown on the top. Two moderate (*EIF4a*) to highly (*EF1-α*) expressed housekeeping genes are shown as controls to verify equal expression across tissues. The map is based on log2-transformed RPKM values (blue represents weakly expressed genes, and red represents strongly expressed genes).

Expression of BP76

We profiled the expression of the six laccase genes in *N. taracua* by aligning the Illumina 100-bp sequence tags from the blue and white worker tissue samples with the TA-contig sequences. Quantitative RNAseq analysis of the transcripts revealed that most of the laccases (laccases 2, 3, 4, 5) were expressed at low level, while laccase-1 was expressed at moderate but comparable levels across all tissues sampled. In contrast, the candidate laccase BP76 identified at the protein level displayed variable but clear tissue-specific expression levels. BP76 is highly expressed in blue workers compared with white workers, with the highest overall level of expression in the crystal glands of blue workers, followed by the crystal glands of white workers (fig. 2). Overall, the expression patterns of the identified laccases verified a single highly abundant laccase (BP76) expressed in crystal glands of blue workers. The laccase gene expression data obtained by RNAseq was verified using replicated quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) (see supplementary fig. S4, Supplementary Material online).

Labial Gland Secretions

The dissected labial glands of blue workers contain three mildly toxic major compounds: the dominant 2-methyl-hydroquinone, along with 2-ethyl-hydroquinone, and hydroquinone, while these compounds are not detected in labial glands of white workers (fig. 3). The bursting liquid of blue workers contains large amounts of the highly toxic 2-methyl-*p*-benzoquinone (along with 2-ethyl-*p*-benzoquinone and *p*-benzoquinone), whereas only traces of the same compounds were found in the bursting liquid of white workers (fig. 3). In addition, the amounts of *p*-benzoquinone, 2-methyl-*p*-benzoquinone, and 2-ethyl-*p*-benzoquinone are low in the labial glands of blue workers but sharply increase after autothysis. Thus, mildly toxic hydroquinones accumulate in the labial glands of blue workers, and are turned into highly toxic benzoquinones in the bursting liquid following autothysis, whereas the amounts of hydroquinones and benzoquinones are low in white workers.

Enzymatic Assay

To confirm that BP76, a putative laccase, has phenol oxidase activity and thus participates in the oxidation of hydroquinones to benzoquinones, we performed a standard functional test. Solubilized *N. taracua* blue crystals possess significant enzymatic activity towards a panel of model phenol oxidase substrates (table 1). The efficient conversion of *p*-diphenol substrates, *o*-diphenols, 2,6-dimethoxyphenol, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) supported that the tested enzyme exhibits substrate specificity of laccases (Baldrian 2006). "In gel" zymography analysis confirmed that BP76 is the only component of *N. taracua* blue crystals responsible for the detected laccase-like activity (see supplementary fig. S5, Supplementary Material online). Furthermore, we investigated the sensitivity of BP76 toward a range of known inhibitors and found effective inhibition by several selective inhibitors of laccases but also tyrosinases (table 2). The enzymological analyses demonstrated laccase-like activity of BP76 and provided functional evidence supporting the proteomic identification of BP76 as a laccase, although BP76 also depicts enzymological activities similar to tyrosinase.

Discussion

In this study, we identified and characterized both constituents of the activated two-component defense system of old *N. taracua* workers. Unlike all other termite defensive systems, the *N. taracua* worker defense involves two distinct structures, the crystal glands and the labial glands, whose products interact after sacrificial autothysis (Šobotník et al. 2012, 2014). Our results show that the crystal glands secrete BP76, a multicopper protein from the laccase family with phenol oxidase activity (Solomon et al. 1996, 2014). The analyses of the labial glands and bursting liquid allowed tracing conversion of hydroquinones into their benzoquinone analogues, which are well-known for their toxic properties exploited by many arthropods (Bettini 1978; Eisner et al. 2005).

Laccases form a large protein family with representatives in bacteria, plants, fungi, and animals including insects. They generally reveal a very broad range of natural substrates, being able to oxidize diphenols, amino- and methoxy-substituted phenols, and aromatic diamines (Dittmer and Kanost 2010). In insects, laccases are involved in diverse processes, ranging from cuticle tanning to detoxification and digestion. For example, they are a component of the venom of the parasitoid wasp *Pimpla hypochondriaca*, probably suppressing immune response of the host (Parkinson et al. 2001, 2003),

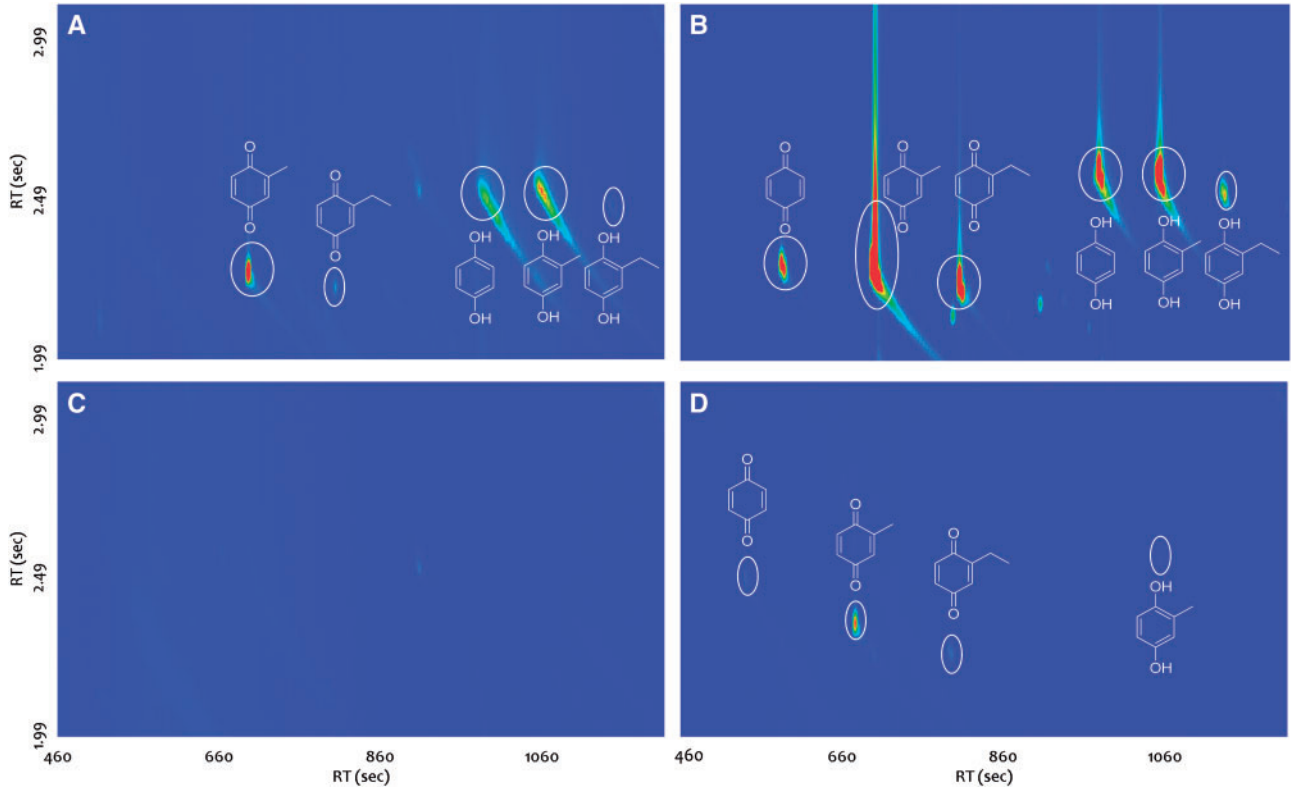

Fig. 3. GC × GC/TOF-MS analyses of (*A*) labial glands of blue workers; (*B*) the bursting liquid of blue workers; (*C*) labial glands of white workers; and (*D*) the bursting liquid of white workers.

Table 1. Enzymatic Activity of BP76 toward Phenol Oxidase Substrates.

Substrate	Activity (units)[a]
ABTS	6.6 ± 0.013
DMP	6.0 ± 0.11
Catechol	72.0 ± 1.21
4-*tert*-Butylcatechol	11.6 ± 0.49
Hydroquinone	$(108 \pm 1.1) \times 10^{-4}$
Methyl-*p*-benzoquinone	$(142 \pm 6.0) \times 10^{-4}$

[a]One unit of activity corresponds to the formation of 1 µmol of product per minute by 1 µg of protein from *Neocapritermes taracua* blue crystals. The mean values ± SD ($n = 3$) are given.

Table 2. Inhibition of Enzymatic Activity of BP76 by Selective Phenol Oxidase Inhibitors.

Inhibitor	Target Enzymes	Inhibitor Concentration (mM)	Remaining Activity (%)[a]
4-Hexylresorcinol		0.5	74.4 ± 0.1
	Tyrosinases	1	62.2 ± 0.2
		4	0
Salicylhydroxamic acid		0.5	54.5 ± 0.1
	Tyrosinases	1	44.7 ± 0.5
		4	26.5 ± 0.2
Cetyltriammonium bromide		0.5	100 ± 0.5
	Laccases	1	60.3 ± 0.2
		4	38.0 ± 0.6

[a]The remaining enzymatic activity of *Neocapritermes taracua* blue crystals is expressed relative to the uninhibited control (100%) and was determined using the activity assay with DMP as substrate. The mean values ± SD ($n = 3$) are given.

and are expressed in the salivary glands of the leaf-hopper *Nephottetix cincticeps*, in which their function is probably related to plant compound detoxification (Hattori et al. 2005). Although laccases were also shown to have digestive functions in *R. flavipes* (Coy et al. 2010; Scharf et al. 2011), they generally participate in tanning of soft pro- or endo-cuticle in many insects (Arakane et al. 2005, 2009; Moussian 2010), including termites (*Reticulitermes speratus*: Masuoka et al. 2013). Our phylogenetic analysis showed that the identified laccases of *N. taracua* belong to distinct clusters, and that BP76 is a member of a termite-specific cluster. This cluster also contains a phenol-oxidizing laccase which was previously found to be expressed in the salivary glands of termite workers and play a role in lignocellulose digestion (Coy et al. 2010). The exclusive placement of the *N. taracua* candidate BP76 within a group of termite sequences, including presumably duplicated *Zootermopsis* genes but excluding other insect laccases, supports a distinct evolutionary history of this termite-specific group. The acceptance of a wide range of substrates as well as the involvement in a diverse set of functions in insects likely increases the probability to gain a new function, for example, after gene duplication events. The main requirement would then be its expression at the right place and in adequate amounts, a change which likely results in

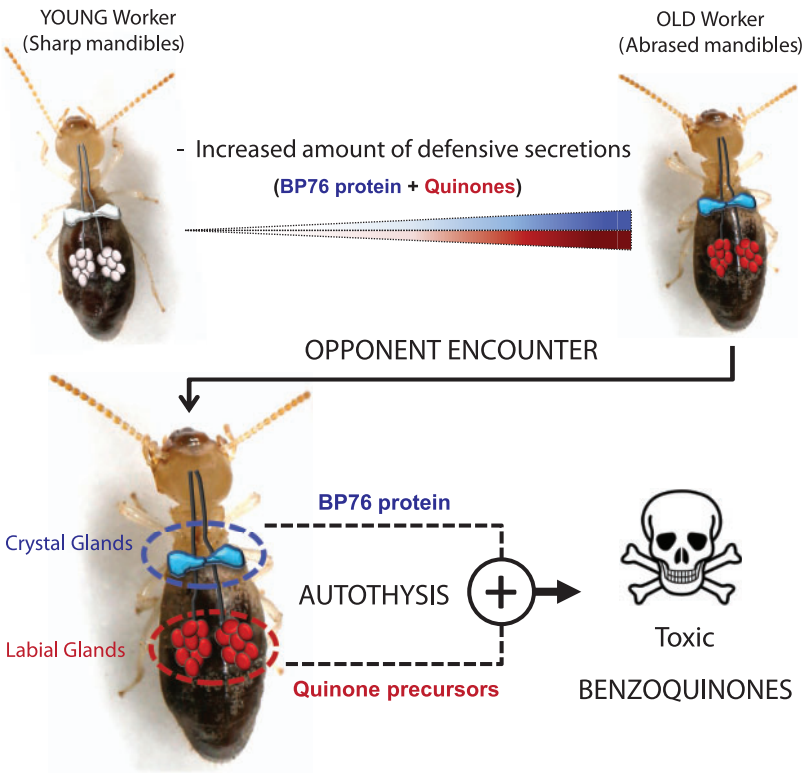

Fig. 4. Schematic drawing of the two-component suicidal weapon occurring in *Neocapritermes taracua* workers.

little extra costs but sometimes important benefits. The high level expression of BP76 in crystal glands of old workers fulfills these requirements.

Although proteins are not common constituents of defensive exudates, some may become sticky after air exposure, and that is why they form defensive secretions in some termites (Moore 1968; Wood et al. 1975), cockroaches (Plattner et al. 1972), and other Arthropods (Deslippe et al. 1996). The same phenomenon was also observed in *N. taracua*, in which the blue crystals turn into extremely sticky material shortly after coming into contact with the hemolymph, and this feature explains incapacitating properties of bursting liquid on house fly (Šobotník et al. 2012).

The crystal gland of *N. taracua* workers is located just below the cuticle of the first abdominal tergite (Šobotník et al. 2014), suggesting that the production of cuticular laccases that oxidize quinones was co-opted as a defensive device. Moreover, this specific position allows BP76 to get into contact with oxygen from the air which is generally required as a second substrate for the enzymatic activity of all laccases. Traces of benzoquinones are also found in the dissected labial gland samples, because the oxidization of hydroquinones takes place at air exposure during sample handling, but the external enzyme BP76 is required for a quick conversion.

Benzoquinones are reactive compounds produced for defense against pathogens or predators in many insects (Bettini 1978; Eisner et al. 2005; Otti et al. 2014), as well as in the termites, such as *Mastotermes* and several Macrotermitinae, in which they are produced in the labial glands of soldiers (Moore 1968; Wood et al. 1975; Sillam-Dussès et al. 2012). In comparison, hydroquinones are less toxic because of their lower electrophilicity resulting in lower reactivity (O'Brien 1991). The mode of production of benzoquinones in *N. taracua* workers is therefore remarkable, as mildly toxic hydroquinones are stored inside the body in anoxic conditions and are converted into benzoquinones only after body rupture by the laccase BP76 (fig. 4). This two-component mechanism recalls the astounding apparatus of bombardier beetles, which produce a mixture of hydroquinone and hydrogen peroxide that, when the beetles are disturbed, react and produce a spurt of boiling benzoquinone and water mixture that can severely harm the opponents (Aneshansley et al. 1969; Arndt et al. 2015).

In *N. taracua*, the efficiency of the two-component activated defense system is achieved by two sets of glands, whose contents need to come in contact by autothysis in order to allow the activation (fig. 4). If the autothysis is not triggered, the contents are not mixed and the worker remains safe. This allows the workers to defend against predators by biting first; only upon imminent danger they can trigger the two-component system, activating their toxic weaponry. While the workers will die after autothysis, the few minutes before death allow them to search for opponents, contaminating them with toxic benzoquinones. *N. taracua* belongs to the most apical family of termites, the Termitidae (Bourguignon et al. 2015), that evolved the greatest diversity of defense strategies, and we can assume that the complex two-component suicidal weapon of *N. taracua* contributed to its ecological success, making it an abundant termite species in many forests in Amazonia.

Materials and Methods

Termites

Workers were repeatedly extracted from several nests of *N. taracua* (Krishna and Araujo 1968) collected in French Guiana, near Petit Saut dam (N 05°04′, W 052°59′), between 2010 and 2013. The nest cores were transported to Prague, and kept alive in a glass box with moist decayed leaf-litter.

Tissue Dissection and RNA Extraction

We performed RNA extraction on tissue of *N. taracua* blue and white workers from three different body parts: 1) whole worker body without gut, labial glands and crystal glands, (40 termites); 2) labial glands (60 termites), and 3) crystal glands (100 termites)) stored in TRIzol (Invitrogen) at −80 °C prior to RNA extraction. The collected tissue material was divided into three different replicates each, and total RNA was extracted using standard phenol–chloroform procedure with TRIzol according to the manufacturer's protocol (Life Technologies), followed by digestion of DNA contaminants with TURBO DNase (Ambion) at 37°C for 1 h and subsequent purification using the RNAeasy Mini Kit (Qiagen) according to the manufacturer's protocol for RNA cleanup. The integrity of the RNA was verified using an Agilent 2100 Bioanalyzer and a RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA). The quantity of RNA was determined using a Nanodrop ND-1000 UV/Vis spectrophotometer (Thermo Scientific). For each of the six samples, equal amounts of total RNA isolated from the three replicates were pooled for transcriptome sequencing.

Illumina Sequencing, TA, and Annotation

Tissue-specific transcriptome sequencing of the six different RNA samples was performed with poly(A) + enriched mRNA fragmented to an average of 150 nt. Sequencing was carried out by the Max Planck Genome Center Cologne (MPGCC) on an Illumina HiSeq2500 Genome Analyzer platform using paired-end (2 × 100 bp) reads. This yielded approximately 30 million reads for each of the six samples. Quality control measures, including the filtering of high-quality reads based on the score given in fastq files, removal of reads containing primer/adaptor sequences and trimming of read length, were carried out using CLC Genomics Workbench v6.5 (http://www.clcbio.com). The de novo TA was carried out with the same software, combining all of the six RNAseq samples, and selecting the presumed optimal consensus transcriptome as described in (Vogel et al. 2014). The resulting final de novo reference TA (backbone) of *N. taracua* contained 96,217 contigs with a N50 contig size of 1,372 bp and a maximum contig length of 23,644 bp. The transcriptome was annotated using BLAST, Gene Ontology and InterProScan searches using BLAST2GO PRO v2.6.1 (www.blast2go.de; Götz et al. 2008) as described in Vogel et al. (2014). To identify candidate genes expressed in the *N. taracua* crystal glands, we established a reference set of known or predicted insect-derived laccases and phenoloxidases using published sequences and searching our in-house database as well as public databases (NCBI). We have deposited the complete short read (Illumina HiSeq2500) data with the following accession numbers: PRJEB11456 (SRA). The complete study can also be directly accessed here: http://www.ebi.ac.uk/ena/data/view/PRJEB11456.

Cloning and Sequencing of BP76 cDNA

Total RNA isolated from *N. taracua* blue crystal glands was transcribed into cDNA using the SuperScript III First-Strand Kit (Invitrogen) according to the manufacturer's protocol. To amplify the cDNA sequence of *N. taracua* laccase BP76, gene-specific primers were used which were designed based on the contig identified from our RNAseq data and the desired product amplified by PCR. Positive PCR bands of the correct size were cut out from the agarose gels, column purified (Zymogen), ligated into the pCR II TOPO vector (Invitrogen). Ligations were transformed into *Escherichia coli* ELECTROMAX DH5α-E electro-competent cells (Invitrogen). Plasmid minipreparation from bacterial colonies grown in 96 deep-well plates was performed using the 96 robot plasmid isolation kit (Nextec) on a Tecan Evo Freedom 150 robotic platform (Tecan). Sequencing of both the 3′- and 5′-termini of as well as with internal primers was carried out on an ABI 3730 xl automatic DNA sequencer (PE Applied Biosystems). Vector clipping, quality trimming and sequence assembly was done with the Lasergene software package (DNAStar Inc.). The full-length cDNA as well as the deduced protein sequence of *N. taracua* laccase BP76 was submitted to Genbank (Accession Number: KT945242).

Sequencing of BP76 Protein Fragments

Blue crystals were extracted from frozen specimens, solubilized in 20 mM Tris-HCl pH 7.0 containing 0.1 M NaCl and clarified by microfiltration. Aceton-precipitated proteins were separated on 15% SDS–PAGE gel, electroblotted onto polyvinylidene fluoride (PVDF) membrane, and visualized with Coomassie blue. The BP76 band was cut out of the gel and blot for mass spectrometric and N-terminal sequencing analysis, respectively. For internal protein sequencing of BP76, the solubilized blue crystals were digested with Lys-C (Roche), resulted fragments separated by SDS–PAGE and analyzed by Edman sequencing as above using a Procise 494 cLC protein sequencer (Applied Biosystems). For mass spectrometric characterization, the BP76 band was digested with trypsin and analyzed by TripleTOF 5600 system (Sciex, Concord, Canada) coupled with Ultimate 3000 RSLC nano system (Thermo Scientific). The top20 MS method was applied. The resulted MS spectra were searched with Protein Pilot 4.5 (Sciex) against NCBI protein database (January 2013) limited to taxonomy: *Insecta*. Peptides identified by Edman sequencing and LC-MS/MS are in supplementary material, Supplementary Material online.

To enable a precise identification of BP76 utilizing our transcriptome database, we used an MS-E approach. Lyophilized proteins from *N. taracua* blue crystal gland secretions were solubilized in 10 mM Tris (pH 8.0) containing protease inhibitor cocktail (1 ×, Pierce). Heat denatured protein samples were separated on a Criterion 4–12% gradient

polyacrylamide SDS–PAGE gel (BioRad) and stained using Colloidal Coomassie blue. The single dominant protein band (see supplementary material, Supplementary Material online) was excised from the Coomassie-stained gel and tryptic digestion and extraction of tryptic peptides from gel pieces was carried out as described before (Shevchenko et al. 2006). For LC-MS, analysis samples were reconstructed in 10 µl aqueous 0.1% formic acid.

The samples were analyzed using a nano Acquity nano-UPLC system on-line connected to a Q-ToF Synapt HDMS mass spectrometer (Waters, Milford). Desalting of samples was performed using a Symmetry C18 trap-column (20 × 0.18 mm, 5 µm particle size, Waters, Milford) at a flow rate of 15 µl/min followed by peptide separation on a nano Acquity C18 analytical column (200 mm × 75 µm ID, C18 BEH 15 130 material, 1.7 µm particle size, [Waters, Milford]). Mass spectrometer settings were as described previously (Kirsch et al. 2012). LC-MS data were acquired in positive ESI mode under data-independent acquisition (MSE) controlled by MassLynx v4.1 software. The collision energy was set at 4 eV in low energy (MS) scans, and ramped from 15 to 40 eV in elevated energy (MSE) scans. The mass range (m/z) for both scans was 300–1,900 and 50–1,700 Da, respectively. The scan time was set at 1.5 s for both modes of acquisition with an interscan delay of 0.2 s. A reference compound, human Glu-Fibrinopeptide B (650 fmol/ml in 0.1% formic acid/acetonitrile [vol/vol, 1:1]), was infused continuously through a reference sprayer for external calibration.

ProteinLynx Global Server version 2.5.2 (Waters, Milford) was used for processing of raw files and for database searching. The continuum LC-MSE data were lock-mass-corrected, smoothed, background-subtracted, centered, deisotoped, and charge-state-reduced. Thresholds for low/high energy scan five ions and peptide intensity were set at 150, 30, and 750 counts, respectively. Processed data were searched against the Swissprot database (downloaded on July 27, 2011, from http://www.uniprot.org/) combined with *N. taracua* protein subdatabase constructed from *N. taracua* transcriptome database by their translation from all six reading frames. Database searches were performed at 2% false discovery rate, using the following parameters: minimum number of product ion matches per peptide (5), minimum number of product ion matches per protein (7), minimum number of peptide matches (2), and maximum number of missed tryptic cleavage sites (1).

Sequence Alignment and Phylogenetic Analysis

Amino acid sequences were aligned using MAFFT version 7.017 (E-INS-I parameter set; Katoh et al. 2002) with default parameters using deduced amino acid sequences from insect laccase transcripts retrieved from *N. taracua* and NCBI and manually inspected for regions of high-quality alignment. MEGA6 (Tamura et al. 2013) was used to select the best-fit substitution model for the amino acid alignment. The unrooted phylogenetic tree was inferred by the ML method using PhyML (Dereeper et al. 2008) available at LIRMM (http://www.phylogeny.fr/) and displayed and edited with FigTree (http://tree.bio.ed.ac.uk/software/figtree). For gene tree generation using Bayesian inference analysis implemented in Mr. Bayes 3.2.2 (Ronquist and Huelsenbeck 2003), the prior was set for the amino acid models to mix, thereby allowing jumps between fixed-rate amino acid models. The *Z. nevadensis* KDR20236 sequence was used as outgroup. The Markov Chain Monte Carlo runs were carried out for 1,000,000 generations. Log likelihood values showed that equilibrium had been reached after the first 10,000 generations which were discarded as "burnin." Two runs were conducted per data set showing agreement in topology and likelihood scores. The ML and the Bayesian tree topologies including their general subfamily relationships were generally in good agreement.

Digital Gene Expression Analysis

Digital gene expression analysis was carried out by using QSeq Software (DNAStar Inc.) to remap the Illumina reads from all six samples onto the reference transcriptome and then counting the sequences to estimate expression levels, using previously described parameters for read mapping and normalization (Vogel et al. 2014). Biases in the sequence data sets and different transcript sizes were corrected using the RPKM algorithm (reads per kilobase of transcript per million mapped reads) to obtain correct estimates for relative expression levels. To control for the effect of global normalization using the RPKM method, we also analyzed a number of highly conserved housekeeping genes, including several genes encoding ribosomal proteins (rpl3, rpl5, rpl7a, rps3a, rps5, rps8, rps18, and rps24), NADH-dh, elongation factor 1alpha and eukaryotic translation initiation factors 4 and 5. The overall expression levels across samples and treatments for these housekeeping genes was lower than 1.3-fold between samples (based on log2 transformed RPKM values), indicating they were not differentially expressed.

Quantitative Real-Time Reverse Transcription PCR analysis (qRT-PCR)

The differential expression of Laccase-1,-2,-5 and laccase BP76 identified by RNAseq was confirmed by qRT-PCR using primers designed based on the contig data (supplementary table S1, Supplementary Material online) using Primer3Plus (http://primer3plus.com/cgi-bin/dev/primer3plus.cgi). Primers were tested by generating individual melting curves and by preparing dilution series for primer efficiency calculations. Primers with a single peak in the melting curve and an efficiency value between 0.9 and 1.1 were used in subsequent quantitative PCR experiments. Reverse transcription and real-time PCR were carried out on three biological replicates of the different *N. taracua* tissue samples using the Verso cDNA synthesis kit and the Absolute Blue qPCR SYBR Green Mix (ThermoFisher) on the CFX Connect Real-Time PCR Detection System (Biorad) according to the manufacturer's recommendations. The data were normalized against *Actin* and *RPS8*, internal controls stable across all samples. Relative expression level differences were calculated based on the ΔΔCT method. The resulting values were then used to calculate the relative

expression levels between the White-Body sample (set to 1) and all other tissue samples relative to the White-Body sample.

Identification of the Labial Gland Chemicals and Defensive Secretion

Labial glands of three categories of workers (white, white/blue intermediate, blue) were dissected from ice-anesthetized individuals and submerged in methanol (4 µl/individual), ultrasonicated for 10 min and extracted at 4 °C overnight. The methanol extracts were analyzed by GC-MS (quadrupole DSQ II, Thermo Scientific) with a nonpolar ZB-5MS column (30 m, id 0.25 mm, 0.25 µm phase thickness). The temperature program was from 50°C to 120°C at 8°C/min rate and then to 320°C at 15°C/min rate. Helium was used as a carrier gas at a constant flow rate of 1 ml/min.

For the analyses of defensive secretion, 10 blue, 10 white and 10 intermediate workers were repeatedly forced to explode by tweezer pressure. The volatiles were extracted from 1.5 ml vials by headspace SPME (yellow SUPELCO SPME fiber, PDMS, 30 µm, non-bonded) for 5 min. Chemical analyses were performed using two-dimensional gas chromatography coupled with mass spectrometric detection (GCxGC/TOF-MS; LECO, Pegasus 3D). The temperature program for the primary column (nonpolar ZB5-MS: 30 m, id 0.25 mm, 0.25 µm phase thickness) was 50 °C (1 min) to 320 °C (4 min) at 8 °C/min; the secondary column (polar RTX-50: 2 m, id 0.1 mm, 0.1 µm phase) was set 10 °C higher. Methanol extracts of 1 µl were injected, representing about 1/10 of worker equivalent.

The identification of particular compounds was based on a comparison of their fragmentation patterns with MS library (NIST MS Search 2.0), and by comparison with commercially available standards (only hydroquinone and methyl-hydroquinone; purchased from Sigma-Aldrich). The calibration was done using methyl-hydroquinone as an external standard.

Enzymatic Essay

Phenol oxidase activity was assayed at 25 °C using 2 mM of the following substrates: ABTS, catechol, 4-*tert*-butylcatechol (TBC), hydroquinone, and 2,6-dimethoxyphenol (DMP). The conditions for each substrate were as follow: 1) 2 mM ABTS ($\varepsilon_{418} = 36{,}000\ M^{-1}cm^{-1}$) in 0.1 M acetate buffer, pH 5.0. Oxidation of ABTS was followed by absorbance increase at 418 nm ($\varepsilon_{418} = 36{,}000\ M^{-1}cm^{-1}$) (Palmieri et al. 1997). 2) 2 mM catechol or 2 mM 4-*tert*-butylcatechol (TBC) in 0.1 M phosphate buffer, pH 7.0. Oxidation was followed by the absorbance increase at 400 nm ($\varepsilon_{400} = 1{,}450\ M^{-1}cm^{-1}$ for quinone product, $\varepsilon_{400} = 1{,}200\ M^{-1}cm^{-1}$ for *o*-*tert*-butylquinone product) (Lang et al. 2012). 3) 2 mM hydroquinone ($\varepsilon_{248} = 22{,}000\ M^{-1}cm^{-1}$) or methyl-*p*-benzoquinone ($\varepsilon_{248} = 21{,}000\ M^{-1}cm^{-1}$) in 50 mM phosphate buffer, pH 5.0. Oxidation was followed by an absorbance increase at 248 nm. 4) 2 mM DMP in McIlvaine's buffer adjusted to pH 5. Oxidation of DMP was followed by an absorbance increase at 477 nm ($\varepsilon_{477} = 14{,}800\ M^{-1}cm^{-1}$) (Li et al. 2012). The reaction mixture consisted of 100 µl of the substrate with 5 µl of aqueous sample solution (0.15 µg protein/µl), and the oxidation of the substrate was monitored without enzymes and under atmospheric condition. The oxidation reaction was followed by the absorbance increase at specific wavelength allowing detection of the newly formed product. Enzymatic activity was always expressed in international units per minute, that is the number of µmol product released by 1 µl of enzyme solution per minute at 2 mM of substrate concentrations (Lang et al. 2012). Data are expressed as mean ± SD ($n = 3$).

We also tested the activity of BP76 using "in gel" zymography. Native PAGE was performed using 5% and 8% polyacrylamide for the stacking and resolving gels, respectively. Electrophoresis was carried out at 150 mV at 4°C. After electrophoresis the slab gels were subjected to the activity staining, the gel was first soaked in 30 ml of 50 mM TrisHCl (pH 8.0) for 15 min, and then stained in 50 mM TrisHCl buffer (pH 8.0) containing 0.02 mM DMP and 0.2 mM $CuSO_4$ at room temperature for 15 min. The bands of proteins that were associated with DMP activity were seen as red bands on a white background. All results were recorded by scanning.

We also tested the effect of laccases and tyrosinase inhibitors on BP76 using DMP as substrate. The inhibitors were 4-hexylresorcinol and salicylhydroxamic acid, dissolved in dimethyl sulfoxide at a final concentration of 3.3%, and cetyltriammonium bromide, dissolved in phosphate buffer at pH 7. First, 0.98 ml of 2 mM DMP substrate was mixed with 0.02 ml of different concentrations of inhibitors. The mixture was incubated at laboratory temperature for 30 min, then 0.005 ml (0.15 µg protein/µl) of aqueous sample solution was added to the mixture and the linear increase in absorbance at 477 nm ($\varepsilon_{477} = 14{,}800\ M^{-1}cm^{-1}$) was monitored.

Supplementary Material

Supplementary material is available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

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