Combination of target site mutation and associated CYPs confers high-level resistance to pyridaben in *Tetranychus urticae*

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ABSTRACT

Pyridaben is a mitochondrial electron transport complex I inhibitor. The H110R mutation in the PSST subunit has been reported as a major factor in pyridaben resistance in the two-spotted spider mite, *Tetranychus urticae*. However, backcross experiments revealed that the mutant PSST alone conferred only moderate resistance. In contrast, inhibition of cytochrome P450 (CYP) markedly reduces resistance levels in a number of highly resistant strains. It was reported previously that maternal factors contributed to the inheritance of pyridaben resistance in the egg stage, but the underlying mechanisms have yet to be elucidated. Here, we studied the combined effects of the PSST H110R mutation and candidate *CYP*s, as metabolic resistance factors, on pyridaben resistance in *T. urticae*. We found that the maternal effects of inheritance of resistance in the egg stage were associated with CYP activity. Analysis of differential gene expression by RNA-seq identified *CYP392A3* as a candidate causal factor for the high resistance level. Congenic strains, where the alleles of both *PSST* and *CYP392A3* were derived from a resistant strain (RR_i; i = 1 or 2) and a susceptible strain (SS_i) in a common susceptible genetic background, were constructed by marker-assisted backcrossing. RR_i showed upregulation of *CYP392A3* and high resistance levels (LC_{50} > 10,000 mg L^{-1}), while SS_i had LC_{50} < 10 mg L^{-1}. To disentangle the individual effects of *PSST* and *CYP392A3* alleles, we also attempted to uncouple these genes in RR_i. We conclude that given the variation in LC_{50} values and expression levels of *CYP392A3* in the congenic and uncoupled strains, it is likely that the high pyridaben resistance levels are due to a synergistic or cumulative effect of the combination of mutant *PSST* and associated *CYP*s, including *CYP392A3*, but other yet to be discovered factors cannot be excluded.

Keywords: genetic linkage, acaricide resistance, METI, complex I, Acari, Tetranychidae

1. Introduction

Target site resistance, as well as detoxification, is a major mechanism of pesticide resistance (Feyereisen et al., 2015; Li et al., 2007; Van Leeuwen and Dermauw, 2016; Van Leeuwen et al., 2010). In some cases, resistance levels are strongly correlated with the frequency of target site resistance mutations, such as for I1017F in chitin synthase 1 and etoxazole resistance in the two-spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) (Demaeght et al. 2014; Osakabe et al., 2017; Van Leeuwen et al. 2012). However, a target site mutation alone sometimes has only limited phenotypic strength and cannot explain the high resistance levels observed in the field (Riga et al., 2017).

Pyridaben is a mitochondrial electron transport complex I (NADH:ubiquinone oxidoreductase) inhibitor (METI-I) (Van Leeuwen et al., 2010). METI-Is were introduced commercially in the early 1990s (Hollingworth et al., 1994; Obata et al., 1992) and have been used worldwide. However, *T. urticae* rapidly developed resistance to METI-Is globally (Devine et al., 2001; Kim et al., 2004, 2006; Stumpf and Nauen, 2001). Pyridaben binds to the complex I PSST subunit (Shuler and Casida, 2001), and a nonsynonymous substitution, H110R (H92R in *Yarrowia lipolytica* numbering), in PSST was identified as a resistance factor (Bajda et al. 2017). However, marker-assisted backcross experiments revealed that the mutant PSST alone conferred only moderate resistance (LC_{50} = ~200 mg L^{-1}; Bajda et al., 2017) even though the LC_{50} of the donor Belgium strain, MR-VP, exceeded 30,000 mg L^{-1} (Van Pottelberge 2009).

Inhibition of cytochrome P450 (CYP) activity by piperonyl butoxide (PBO) reduce LC_{50} values for pyridaben from > 10,000 to < 100 mg L^{-1} in several Korean and

74 Japanese (NPR) *T. urticae* strains (Kim et al., 2006; Sugimoto and Osakabe, 2014). In
75 contrast, inhibition of esterase and glutathione *S*-transferase did not affect pyridaben
76 resistance (Kim et al., 2006; Sugimoto and Osakabe, 2014). Differing from these East
77 Asian strains, treatment with PBO and inhibition of esterase by *S,S,S*-tributyl-
78 phosphorotrithioate (DEF) reduced the LC_{50} values from > 35,000 to 385 and 560 mg
79 L^{-1}, respectively, in MR-VP (Van Pottelberge et al., 2009). Taken together, these
80 observations suggest an association of CYPs with high-level pyridaben resistance,
81 whereas the role of esterase in pyridaben resistance may be different between MR-VP
82 and the Korean and Japanese strains.
83 A striking characteristic of pyridaben resistance is the maternal effect in inheritance
84 of resistance, which is limited to the egg stage; no clear maternal effect is observed in
85 other stages (Devine et al., 2001; Stumpf and Nauen, 2001; Sugimoto and Osakabe,
86 2014; Van Pottelberge et al., 2009). In general, maternal effects can be conferred by a
87 target site mutation encoded in mitochondrial DNA (Van Leeuwen et al., 2006; Van
88 Nieuwenhuyse et al., 2009). However, the target-site, PSST, is encoded in the nuclear
89 DNA, and whether additional mitochondrial factors are involved in the mode of action
90 and resistance is unknown. Quantitative trait locus (QTL) analysis of pyridaben
91 resistance based on the crosses between Japanese resistant, NPR, and susceptible, NPS,
92 strains using microsatellites over scaffold 7 revealed a single common QTL region over
93 the loci of *PSST* and five *CYP*s: *CYP385A1* (*tetur07g05500*), *CYP392A1*
94 (*tetur07g06410*), *CYP392A3* (*tetur07g06460*), *CYP392A4* (*tetur07g06480*), and
95 *CYP390B1* (*tetur07g08209*) (Bajda et al., 2017). Therefore, these *CYP*s were
96 hypothesized to contribute to the high levels of pyridaben resistance, and potentially to
97 the maternal effects observed in eggs.

In this study, we further examined the involvement of CYPs, and the effects of the combination of *PSST* H110R mutation and candidate *CYP*s, on pyridaben resistance in *T. urticae*. First, we determined the DNA sequences of mitochondrially encoded complex I subunits ND1 and ND5, to check for polymorphisms and exclude maternal effects due to mitochondrially encoded proteins. Then, we investigated the association of CYP enzymatic activity with the maternal effects in eggs. Candidate *CYP*s associated with pyridaben resistance were further identified based on comprehensive gene expression analysis using RNA-seq and quantitative real-time PCR (qPCR), as well as toxicological experiments. Finally, we evaluated the effects of the combination of H110R mutation in PSST and candidate *CYP* on the resistance level through double introgression of these alleles from resistant strain into a susceptible genetic background and uncoupling of these genes in the congenic lines.

2. Materials and methods

2.1. Acaricide

A commercial formulation of pyridaben (20% FL; Nissan Chemical, Tokyo, Japan) was used for toxicity bioassays.

2.2. T. urticae strains

All *T. urticae* strains were originally collected from agricultural fields in Japan (Table 1). Pyridaben-resistant (NPR) and -susceptible (NPS) strains were the same as those reported previously by Sugimoto and Osakabe (2014) and Bajda et al. (2017), and underwent positive and reverse selection with pyridaben in the laboratory.

Nine other field-collected strains were maintained without pyridaben selection. Seven and one of the nine strains were the same as those described by Osakabe et al. (2017) and Sugimoto et al. (2020), respectively. All strains were reared on kidney bean (*Phaseolus vulgaris* L.) leaf disks (~5 cm in diameter for stock culture) placed on water-soaked cotton in Petri dishes (9 cm in diameter) in the laboratory at 25°C, with 60% relative humidity (RH) and a 16-h light/8-h dark photoperiod (unless otherwise noted).

2.3. Maternal effects of pyridaben resistance inheritance in the F1 egg stage

To obtain F1 eggs, mass mating was performed between 200 unmated females and 200 adult males on kidney bean leaf disks for 24 h. Then, five inseminated females were introduced to each of four kidney bean leaf disks (2 × 2 cm) and allowed to produce eggs for 24 h. The adult females were removed, and the leaf disks were immersed in pyridaben solution for 10 s. After air-drying on a paper towel, the leaf disks were placed on water-soaked cotton in Petri dishes and kept in the laboratory. Seven days later, hatchability of the eggs was assessed under a stereomicroscope.

One Petri dish was assigned to one test concentration of pyridaben including control treatment with water. Mass mating was performed for NPR♀ × NPR♂ (two replicates), NPR♀ × NPS♂ (three replicates), NPS♀ × NPR♂ (two replicates), and NPS♀ × NPS♂ (four replicates). The data of replicates were combined and used to compute LC_{50} values. We limited the highest test concentration to 20,000 mg L^{-1} because the viscosity of pyridaben solution increased with concentration.

Corrected mortality (*Mc*) was calculated using Abbott's formula (Abbott, 1925); $Mc = (B - A)/B$, where A and B represent the survival rates on test and control leaves, respectively. The LC_{50} values and 95% confidence intervals (CIs) were computed by a

program for the 50% effective dose (ED_{50}) using an R script (http://aoki2.si.gunma-u.ac.jp/R/ed50.html; Aoki, 2011) modified by Sugimoto and Osakabe (2014). Statistical significance was evaluated by inspecting overlap of the 95% CIs of LC_{50} values.

2.4. Sequencing of ND1 and ND5 subunits of complex I

Mitochondrial DNA of NPS and NPR strains was extracted from ~100 adult females as described by Van Leeuwen et al. (2008). The ND1 and ND5 genes (*NAD1* and *NAD5*, respectively) were amplified by PCR in a volume of 50 µL (32.3 µL of double-distilled water; 10 µL of buffer; 0.5 mM dNTP-mix; 0.3 µM of each primer; 1.2 µL of template DNA and 1 µL of enzyme-mix) using an Expand Long Range, dNTPack kit (Roche, Basel, Switzerland) with an initial denaturation step at 92°C for 2 min, followed by 35 cycles of 92°C for 20 s, 54°C for 30 s, 58°C for 3 min, and 58°C for 5 min. *NAD1* was amplified in one PCR amplicon, while *NAD5* was amplified in two overlapping amplicons (Table S1). PCR products were purified using an EZNA Cycle Pure Kit (Omega Bio-Tek, Norcross, GA, USA) and sent to LGC Genomics (Berlin, Germany) for Illumina MiSeq paired-end (2 × 300 bp) sequencing (Illumina, San Diego, CA, USA). Generated reads were adapter clipped by LGC Genomics and submitted to the NCBI database (see Accession number section). Next, clipped reads were mapped against the *T. urticae* mitochondrial genome from the London strain (Grbić et al., 2011) using Bowtie 1.1.2 (Langmead et al., 2009) with the default settings. From the resulting BAM file for each strain (NPS and NPR), the *NAD1* and *NAD5* consensus sequences were derived using a Perl script (gene_extractor.pl) written by Rutger Vos (available at https://github.com/naturalis/fastq-simple-tools/tree/master/script) and a gff-file of the London mitochondrial genome, which was

created using the MITOS web server (Bernt et al., 2013). In case heterozygous single nucleotide polymorphisms (SNPs) were present, the SNP with the highest frequency was included in the final consensus sequence.

2.5. Involvement of CYP activity in pyridaben resistance and its maternal effects in eggs

2.5.1. CYP activities in females and eggs

CYP activities were evaluated by *O*-deethylation of 7-ethoxy-4-trifluolomethylcoumarin (7EFC) according to Van Leeuwen et al. (2005), in females and eggs of the NPR and NPS strains, and in the F1 generation derived from their reciprocal crosses. To obtain the F1 generation, 200 NPR and NPS females were mated with 200 males of NPS (RS) and NPR (SR), respectively, on leaf disks (5 × 5 cm) for 24 h. Eggs and adult females of the F1 generation were obtained for subsequent CYP analysis in the same way as for the mass mating in section *2.3*.

Then, 200 adult females or 1,000 eggs (within 1 day after oviposition) were homogenized in 350 µL of sodium phosphate buffer (0.1 M, pH 7.6) at 5°C. After centrifugation (1,000 × g) for 15 min at 4°C, 50 µL of the supernatant was mixed with 50 µL of substrate solution (0.4 mM 7EFC, 0.2 mM $NADP^+$, 1 mM glucose-6-phosphate, and 0.014 units of glucose-6-phosphate dehydrogenase in sodium phosphate buffer) in 96-well microplates. The microplates were shaken automatically at 37°C for 30 min in the dark inside a microplate reader (SH-9000Lab; Corona Electric, Hitachinaka, Japan). The enzyme reaction was stopped by adding 100 µL solution of 1:1 acetonitrile:TRIZMA base solution (0.05 M, pH 10). After 10 min, 7-hydroxy-4-trifluoromethylcoumarin (7HFC) fluorescence was measured at 510 nm with an excitation wavelength of 410 nm. Wells containing sodium phosphate buffer instead of

188 the supernatant were used as negative controls. The 7EFC-*O*-deethylation activity and
189 protein concentration in the supernatant were calibrated using a 7HFC standard curve
190 (0.02–0.2 μM) and a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham,
191 MA, USA).
192 Three biological replicates with three technical replicates were used in all
193 experiments. In each replicate, measurements of females and eggs of NPR and NPS
194 strains (or F1 from the reciprocal crosses) were performed on the same microplate using
195 7HFC solutions for calibration. The reagents used for CYP analysis were purchased
196 from FUJIFILM Wako Pure Chemical (Osaka, Japan), Sigma-Aldrich Japan (Tokyo,
197 Japan), or Oriental Yeast (Tokyo, Japan).
198 Differences in CYP activity according to resistance status were evaluated by one-
199 way analysis of variance (ANOVA), assuming that the samples in the same microplate
200 (replicate) were paired observations, using the "aov" module after Bartlett's test
201 (performed with the "bartlett.test" module). All statistical analyses in the following
202 sections were performed using R software (R Foundation for Statistical Computing,
203 Vienna, Austria) (R Core Team, 2017).

204 *2.5.2. Effects of CYP inhibition in females on the resistance status of their eggs*
205 Fifty adult females of NPR were introduced onto four leaf disks. After 30 min, two
206 leaf disks each were sprayed with 2 mL of solvent (acetone:water = 1:1) with or without
207 PBO (PBO+ and PBO−, respectively) (250 mg L^{-1}; FUJIFILM Wako Pure Chemical).
208 The females were allowed to feed on the leaves for 4 h after the solvent had dried. From
209 each of the four leaf disks, 40 females were moved to eight leaf disks (2 × 2 cm; 5 ♀♀
210 per leaf disk). After oviposition for 24 h, all females were removed from the leaf disks.

Eight leaf disks from the PBO+ (or PBO−) treatment group were immersed in 10,000 mg L^{-1} pyridaben solution for 10 s (PBO+/Pyr+ [or PBO−/Pyr+]). The remaining eight leaf disks of PBO+ (or PBO−) were immersed in water for 10 s (PBO+/Pyr− [or PBO−/Pyr−]). Egg hatchability was assessed under a stereomicroscope after 7 days. The experiments for these treatments were performed with six replicates.

The numbers of hatched and unhatched eggs of leaf disks in each treatment group were combined for each replicate and transformed into empirical logits. The effects of PBO and pyridaben treatments on egg hatchability were evaluated by two-way ANOVA using the "aov" module after Bartlett's test (performed using the "bartlett.test" module). Multiple comparisons among the four treatments were performed by the Tukey-Kramer method using the "TukeyHSD" module, with one-way ANOVA model constructed using the "aov" module.

2.6. RNA-seq of pyridaben-resistant and -susceptible strains

Two hundred adult females of NPR (or NPS) were introduced onto each of two leaf disks. After 24 h, one of the two leaf disks was immersed in pyridaben solution (200 mg L^{-1}) for 10 s to induce resistance-associated gene expression (induced samples). The other leaf disk was immersed in water without pyridaben for 10 s as a control (non-induced samples). After 1 h, mites were collected and homogenized in 800 μL of Isogen (Nippon Gene, Tokyo, Japan). Total RNA samples were purified in accordance with the manufacturer's protocol. After isopropanol precipitation, total RNA was dissolved in 100 μL of nuclease-free water (Qiagen, Hilden, Germany). RNA-seq (paired-end; read length: 101 bp) was performed by Hokkaido System Science Co. (Sapporo, Japan) using an Illumina HiSeq2000 (Illumina). Three biological replicates were prepared and

234 analyzed for each strain and treatment.

235 The raw RNA-seq reads of each sample were cleaned using Trimmomatic 0.32
236 (Bolger et al., 2014). A reference transcriptome assembly was constructed by de novo
237 assembly of merged clean reads (six replicates) of the NPR strain using Trinity version
238 r20140717 (Haas et al., 2013). Annotation of the reference transcriptome assembly and
239 calculation of gene expression levels in each strain were performed as described by
240 Sugimoto et al. (2020). Differentially expressed genes (DEGs) between strains and
241 between treatments were determined using the iDEGES/edgeR method in TCC version
242 1.1.99 (Sun et al., 2013), with the following criteria: false discovery rate (FDR) < 0.05,
243 and M-value $[\log_2(\text{NPR}) - \log_2(\text{NPS})] \geq 1$. Raw tag count data of three replicates in
244 each condition (strain and treatment) were examined by the iDEGES/edgeR method for
245 each comparison (normalization of the gene expression levels was performed internally
246 using the iDEGES/edgeR method).

247 SNP calling of the PSST gene in each strain was performed by mapping the clean
248 reads to the sequence of the PSST transcript in the assembly using SAMtools 0.1.19 (Li,
249 2011).

250 *2.7. Effects of PSST mutation and CYP expression level on pyridaben susceptibility in*
251 *local populations*

252 *2.7.1. Toxicological bioassay*

253 The pyridaben susceptibilities of 11 *T. urticae* strains (Table 1) were tested using
254 the spraying method of Kunimoto et al. (2017). Twenty-five adult females were
255 introduced onto a leaf disk (3 × 3 cm) and sprayed with pyridaben solution (1 mL) at the
256 assigned concentration (adhesion amount: 1.91 ± 0.16 mg cm^{-2}). The maximum

257 pyridaben concentration was 10,000 mg L^{-1} due to the viscosity of the solution. Milli-Q
258 water (1 mL) was sprayed onto the control leaf disk assigned for control. Mortality was
259 checked after 24 h. Mites that could not walk normally after being touched with a fine
260 brush were counted as dead. Four leaf disks were used for each concentration. The data
261 of the four leaf disks were combined to compute LC_{50} values (see *Section 2.3*).

262 *2.7.2. PSST mutation frequency analysis using qPCR with a resistance allele-specific*
263 *primer set*

264 We predicted the PSST H110R frequency in the 11 local *T. urticae* strains (Table 1)
265 by qPCR using a resistance allele-specific primer set (tu_PSST_222F and
266 tu_PSST330T_357R) and a common primer set as an internal reference (tu_PSST_308F
267 and tu_PSST_419R), developed by Maeoka et al. (2020) (Table S2). DNA samples were
268 extracted from 50 adult females using a DNeasy Blood & Tissue Kit (Qiagen), treated
269 with RNase A (Qiagen), and then purified using the NucleoSpin gDNA Clean-up XS
270 (Macherey-Nagel, Düren, Germany). qPCR was performed using a LightCycler 96
271 System (Roche Diagnostics, Tokyo, Japan) with TB Green Premix Ex TaqTM II (Tli
272 RNaseH Plus) (TaKaRa Bio, Kusatsu, Japan) in a 20-μL reaction mixture
273 (Supplementary information 1). We used three biological replicates with two technical
274 replicates for each strain.

275 For calibration, a strain homozygous for PSST H110R (RR strain) was established
276 from descendants of mating pairs from NPR, based on direct sequencing analysis using
277 a Genetic Analyzer 3130 with BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo
278 Fisher Scientific) (Supplementary information 2) and subjected to qPCR in each
279 replication. H110R frequency was computed by the ΔΔCq method (Livak et al., 2001;

Pfaffl, 2001; Schmittgen et al., 2000; Winer et al., 1999), and based on Osakabe et al. (2017) and Maeoka et al. (2020).

2.7.3. RT-qPCR analysis for validation of CYP expression determined by RNA-seq

Total RNA was prepared from 200 adult females for the 11 strains listed in Table 1 using an RNeasy Micro Kit (Qiagen). First-strand cDNA was synthesized from total RNA using a PrimeScript RT Reagent Kit with gDNA eraser (TaKaRa Bio). Of the 14 *CYP*s shown to be upregulated in NPR by RNA-seq (see *Section 3.4*), we designed efficient and specific primer sets for 12 *CYP*s based on the RNA-seq data; specific primer sets could not be produced for two *CYP*s (*CYP392A10* and *CYP392D8*). The gene expression levels of the 12 *CYP*s were evaluated by qPCR analysis using the LightCycler 96 system with TB Green Premix Ex Taq II (Tli RNaseH Plus) (Supplementary information 1). qPCR was performed for three biological replicates for each strain, with two technical replicates. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ubiquitin genes were used as references (Table S3). The gene expression level was computed with the ΔΔCq method.

The $\log_2$-fold changes (Log2FC = −ΔΔCq) of *CYP* expression level in the strains in comparison to the NPS strain were evaluated by multiple comparison of ΔCq values with Dunnett's contrast, using the "glht" module in the "multicomp" package, by with a two-way ANOVA model without interaction (performed using the "aov" module). Prior to the two-way ANOVA, Bartlett's test was performed for all genes using the "bartlett.test" module.

2.7.4. Regression analysis and principal component analysis (PCA) of the correlation of the PSST mutation and upregulation of CYP genes with pyridaben susceptibility

303 The correlation of Log2FC in PSST mutation frequencies (calibrator: RR; *Section*
304 *2.7.2*) and expression levels of CYP genes (calibrator: NPS) in the 11 strains (*Section*
305 *2.7.3*) with their LC_{50} values (*Section 2.7.1*) were analyzed using the "lm" module. For
306 PCA, the Log2FC data set for each gene was standardized over the 11 strains. PCA for
307 PSST and CYP genes was performed using the "prcomp" module.

308 *2.8. Sanger sequencing for CYP392A3*

309 *2.8.1. Cloning and sequencing of NPR CYP392A3 alleles*

310 Based on the results presented in *Section 2.7*, we cloned and sequenced *CYP392A3*
311 of the NPR strain. To clone *CYP392A3*, total RNA was prepared from 200 adult females
312 of NPR and cDNA was synthesized as described in *Section 2.7.3*. The DNA construct of
313 *CYP392A3* was produced from amplified PCR products (Supplementary information 2)
314 using a primer set for *CYP392A3* (CYP392A3fusion-F and -R; Table S4) designed for
315 cloning the pAc5.1/V5-His A plasmid (Thermo Fisher Scientific). Cloning was
316 performed using an In-Fusion HD Cloning Kit (TaKaRa Bio) according to the
317 manufacturer's protocol. One Shot TOP10 Chemically Competent *Escherichia coli*
318 (Thermo Fisher Scientific) was transformed with the plasmid using a rapid chemical
319 transformation according to the manufacturer's protocol. Four transformed *E. coli*
320 colonies occurred on the L-broth plates containing ampicillin (100 μg mL^{-1}) were
321 separately amplified in 5 mL of L-broth (ampicillin 50 μg mL^{-1}) at 37°C for 24 h, and
322 plasmids were purified using NucleoSpin Plasmid EasyPure (TaKaRa Bio). The purified
323 plasmids were used for sequencing (Supplementary information 2) with the primers of
324 CYP392A3-forward and -reverse, CYP392A3-692F, CYP392A3-938R, and Ac5-seq-F
325 and -R (Table S4).

326 *2.8.2. Direct sequencing of CYP392A3 alleles in males of local strains*

327 To assess the frequency of the *CYP392A3* variant with the NPR specific insertion in
328 the local populations described in *Section 2.8.1* (see also *Section 3.6.1*), we performed
329 direct sequencing of *CYP392A3* for haploid adult males (Supplementary information 2).
330 The numbers of males used for sequencing were 16, 12, 12, 10 11, and 8 for NPS, Izu2,
331 Masu, SoOm1, Yawata, and NPR, respectively.

332 First, we performed sequencing analysis using only CYP392A3-reverse primer for
333 the indel region. The templates of *CYP392A3* used for sequencing were amplified using
334 CYP392A3-forward and -reverse primers (Table S4). Then, seven and four individuals
335 were picked up from NPS, and each of the other strains, respectively, with reference to
336 the substitution. These were subjected to additional sequencing analysis using
337 CYP392A3-forward, CYP392A3-692F, and CYP392-938R primers (Table S4) to
338 complete the amplicon sequences.

339 *2.9. Effects of the combination of mutant PSST and highly expressed CYP392A3*

340 *2.9.1. Double introgression of mutant PSST and CYP392A3 alleles (INS2+ type) into*
341 *susceptible strain by the marker-assisted backcrossing*

342 Next, we performed double introgression of the mutant *PSST* and highly expressing
343 *CYP392A3* genes into the genetic background of the susceptible strain. However, as
344 monitoring gene expression as a marker in each individual mite was difficult, we used
345 an insertion (INS2; see Fig. S8) commonly revealed by *CYP392A3* sequencing of
346 strains with high *CYP392A3* expression levels (see *Section 3.6*) for the following
347 introgression experiments, as an alternative marker.

348 Two paternal lines of NPR (R_1 and R_2; c.329A>G [H110R] in *PSST* and

c.1242_1243insAATATTACC [p.E414_N415insNIT; INS2] [INS2+ type] in *CYP392A3*), and two of NPS (S_1 and S_2; 329A [H110] in *PSST* with no insertion at c.1242_1243 [INS2− type] in *CYP392A3*) (see *Section 3.6.1*), were established from descendants of homogeneous mating pairs of NPR and NPS strains (mutation notation follows the Human Genome Variation Society; http://www.hgvs.org/mutnomen/examplesDNA.html; den Dunnen and Antonarakis 2000). The genotypes of parental mites were determined by direct sequencing analysis (Supplementary information 2) using CYP392A3-forward and -reverse primers for *CYP392A3*, and PSST-73F and PSST-664R for *PSST* (Table S4).

Marker-assisted backcrossing (Bajda et al., 2017; Riga et al., 2017) was performed for two lines derived from two parental matings (S_1♀ × R_1♂ and S_2♀ × R_2♂) on leaf disks (2 × 2 cm) placed in a growth chamber (28°C, 60% RH, and 16-h light/8-h dark photoperiod; Fig. S1). All individuals used for the mating experiments were subjected to genotyping of *PSST* and *CYP392A3* by direct sequencing. Eight F1 hybrid females were separately backcrossed with S_1 (or S_2) adult males. Eight female offspring produced by females heterozygous for both *PSST* (H110/H110R) and *CYP392A3* (INS2−/INS2+) in each generation were used for the next backcrossing until the B6 generation.

Then, 16 mother–son matings between B6 females and their male offspring were performed for each line. The mothers were kept at 10°C during development of their offspring to adulthood. Twelve B7 females from B6 females that were heterozygous for both *PSST* and *CYP392A3*, and mated with sons of H110R and INS2+ (or H110 and INS2−), were used for the next mother–son matings. The B8 offspring from one B7 female fixed to H110R and INS2+ (or H110 and INS2−) were chosen from each line

and propagated as the H110R and INS2+ homozygous subline (RR_1 [or RR_2]) (or H110 and INS2− homozygous subline; SS_1 [or SS_2]) (Fig. S1).

Pyridaben susceptibilities and *CYP392A3* expression levels of the paternal lines and congenic sublines were examined by the toxicological analysis (*Section 2.7.1*) and RT-qPCR analysis (Supplementary information 1), respectively. For RT-qPCR, we designed a new forward primer for *CYP392A3* (5′-AAAGAGCTTTTCAAACACCAGAATG-3′; amplification efficiency E = 1.90), because a nucleotide mismatched to NPS was found in the original forward primer (Table S3; see *Section 2.8.2*). *GAPDH* and *ubiquitin* were used as reference genes.

The expression levels of *CYP392A3* of R_i and RR_i (i = 1 or 2) lines, against the S_i and SS_i lines, respectively, were evaluated based on ΔCq values by two-way ANOVA without interaction (performed using the "aov" module). Differences in Log2FC between R_i and RR_i lines (the calibrators were S_i and SS_i, respectively) were evaluated by two-way ANOVA without interaction using the "aov" module, following the multiple comparison by the Tukey–Kramer method using the "TukeyHSD" module. Prior to the two-way ANOVA, Bartlett's test was performed in all genes using the "bartlett.test" module.

2.9.2. Uncoupling of resistant congenic line (RR_i)-derived PSST (H110R) and CYP392A3 (INS2+ type) alleles

We performed marker-assisted crossing to establish homozygous strains that had either mutant PSST or INS2+ type CYP392A3 (Fig. S2). Genotypes of *PSST* and *CYP392A3* of all mites used in the uncoupling process were monitored by the high-resolution melting (HRM) analysis using a LightCycler 96 system with TB Green

Premix Ex Taq II (Tli RNaseH Plus) (Supplementary information 2). Crude DNA samples of mites, as well as direct sequencing as described in *Section 2.8.2* (Supplementary information 2), were used for HRM. Genotypes of *PSST* and *CYP392A3* of all crude DNA samples associated with the established uncoupled strains in all generations were finally confirmed by the direct sequencing after the crossing experiments had been completed, and no discrepancies were observed.

Twelve unmated F1 females from 12 individual crosses between SS_1 (or SS_2) females and RR_1 (or RR_2) males (two unmated females per pair) laid haploid male F2 (F2M) eggs on leaf disks (1.5 × 1.5 cm) in a growth chamber (28°C, 60% RH, and 16-h light/8-h dark photoperiod). The resulting 84 F2M males (7 per F1 female) were individually mated with SS_1 (or SS_2) females.

Four mating pairs from the SS_2♀ × RR_2♂ line, where the genotypes of F2M were H110R *PSST* and INS2+ type *CYP392A3* (R-INS2+), H110 and INS2− type (H-INS2−), H110R and INS2− type (R-INS2−), and H110 and INS2+ type (H-INS2+), respectively, were selected for mother–son mating (a series of recombinant F2Ms was not obtained in the SS_1♀ × RR_1♂ line; see Table S8). Twelve F3 females (F3F) from each of the four pairs (48 females in total) were mated with their own sons (F4M). Sixteen offspring females (F4F), produced by F3F that mated with F4M of the same genotypes as F2M, were used for the next mother–son matings. The F5 offspring of one F4F homozygote in both *PSST* and *CYP392A3* loci were chosen for each of the four F2M genotypes and propagated as uncoupled sublines.

Toxicological bioassay and RT-qPCR analysis of *CYP392A3* of uncoupled strains were performed and evaluated statistically, as described in *Section 2.7.1* and *2.9.1*, respectively. To compute Log2FC of *CYP392A3*, the expression data of S_1 (i.e., the

lowest expression level) were used as a calibrator, to align the standards for comparison among uncoupled strains.

3. Results

3.1. Maternal effects of pyridaben resistance inheritance in the F1 egg stage

The LC_{50} values of F1 eggs were 1.19, 12.6, 5,916, and 61,310 mg L^{-1} in NPS (♀) × NPS (♂), NPS × NPR, NPR × NPS, and NPR × NPR, respectively (Fig. 1, Table 2), confirming the maternal effects first observed by Sugimoto and Osakabe (2014) for these strains.

3.2. Sequencing ND1 and ND5 subunits of complex I

No sequence polymorphisms were observed between NPR and NPS for *NAD1* (Supplementary information 3). For *NAD5*, the 495th amino acid residue was fixed to leucine (TTA) and methionine (ATA) in NPS and NPR, respectively (Supplementary information 3). The corresponding residue in the London strain (Grbić et al. 2011), which is a pyridaben susceptible strain (Dermauw et al. 2013), is a methionine (M495). Moreover, L495 is not located in the postulated quinone-binding region (*Bos taurus* ND5, Nakamaru-Ogiso et al. 2003; Supplementary information 3), and substitution preference of leucine to methionine is favored in all protein types (Betts and Russell 2003), suggesting that this substitution is much likely not involved in pyridaben resistance in *T. urticae*.

3.3. Involvement of cytochrome P450 activity in the maternal effects on eggs

3.3.1. CYP activities in adult females and eggs

The 7EFC-*O*-deethylation activity was significantly higher in NPR than NPS in eggs, whereas the difference was only marginally significant in adult females (Fig. 2a). In the F1 generation, after reciprocal crosses between NPR and NPS, activity was equivalent between RS and SR in F1 adult females, but was higher in RS than SR eggs (Fig. 2b).

3.3.2. Effects of CYP inhibition in females on the pyridaben resistance of their eggs

Two-way ANOVA indicated significant effects of pyridaben treatment on eggs ($F_{[1, 20]} = 54.617$, $P = 3.88 \times 10^{-7}$) and PBO treatment on females ($F_{[1, 20]} = 5.879$, $P = 0.0249$), and of their interaction ($F_{[1, 20]} = 9.588$, $P = 5.69 \times 10^{-3}$) on egg hatchability. Treatment of adult females with PBO alone (PBO+/Pyr−) did not affect egg hatchability, similar to the group treated without PBO and pyridaben (PBO−/Pyr−) (Fig. 3). However, 81.7% of the eggs produced by PBO-untreated females hatched after pyridaben treatment (PBO−/Pyr+), whereas treatment with PBO combined with pyridaben (PBO+/Pyr+) reduced the egg hatchability to 36.6%, indicating synergistic toxicity.

3.4. RNA-seq of pyridaben resistant and susceptible strains

Of over 24 million reads generated for each replicate, ≥ 91% passed the trimming process (Table S5). The reference transcriptome assembly consisted of 28,406 genes with 39,245 transcripts, and the N50 and E90N50 values of the assembly at the gene level were 2,473 bp and 3,318 bp, respectively (Table S6).

The numbers of up- and downregulated DEGs in NPR compared to NPS were

462 1,544 and 456, respectively, in samples induced with pyridaben, and 1,429 and 405,
463 respectively, in those without induction (Supplementary Data 1). In contrast, no DEGs
464 were detected between samples with and without induction by pyridaben treatment.
465 Sixteen *CYP*s were upregulated in NPR, compared to NPS in either or both of induced
466 and non-induced samples (Fig. 4). Of the five *CYP*s with the highest (> 4) M-values
467 [$\log_2$(NPR) – $\log_2$(NPS)], *CYP392D8* (coverage of gene length aligned with top hit
468 transcript = 11.3%), *CYP392D2* (25.9%), *CYP389C1_1* (14.9%), and *CYP392E4*
469 (14.8%) showed negative A-values [{$\log_2$(NPR) + $\log_2$(NPS)} × 2^{-1}], indicating low
470 expression levels in both NPR and NPS. In contrast, *CYP392A3* (coverage = 78.3%)
471 showed a positive A-value (2.62) with a high M-value (4.75; 26.9-fold). Among the
472 other 11 *CYP*s, nine and two genes showed positive and negative A-values, respectively.
473 Four *CYP*s (*CYP392B1*, *CYP392A10*, *CYP389C12*, and *CYP389C11*) and two *CYP*s
474 (*CYP387A1* and *CYP382A1*) were detected as DEGs in samples with and without
475 pyridaben induction, respectively. Next, we determined 14 of the 16 *CYP*s upregulated
476 in NPR as candidate resistance factors (*CYP387A1* and *CYP382A1* were excluded as
477 these were not detected as DEGs in the mites induced with pyridaben); these were
478 subjected to RT-qPCR validation, as described in *Section 2.7.3*.

479 The SNP introducing the H110R mutation in the PSST gene was detected in all
480 replicates of the NPR strain (Table S7). The rate of resistant (H110R) reads was 100%
481 in the NPR strain and 0% in the NPS strain.

482 Additionally, NADPH-cytochrome P450 reductase was upregulated twice (M-value
483 = 1.04) in NPR than NPS in both induced and non-induced samples (Supplementary
484 Data 1).

3.5. Effects of PSST mutation and CYP expression level on pyridaben susceptibility in local populations

3.5.1. Toxicological bioassay

The LC_{50} values of NPR and NPS were 7,756 and 4.03 mg L^{-1}, respectively (Table 3; Fig. S3). Six local strains (Iwate, Yokote, Izu2, SoKg, SoOm2, and Tsukuba) had significantly lower LC_{50} values than the concentration registered for field use (200 mg L^{-1}). Several individuals of Yokote and SoKg survived at concentrations higher than this concentration (Fig. S3). The LC_{50} of Masu exceeded the field concentration (Table 3). Yawata and SoOm1 strains showed high resistance levels.

3.5.2. PSST mutation frequency and expression levels of CYPs

The *PSST* was fixed to H110 in the five pyridaben-susceptible local strains (Iwate, Izu2, SoKg, SoOm2, and Tsukuba; Fig. S4). The H110R frequencies were 0.06, 0.05, and 0.3 in Yokote, NPS, and Masu, respectively. In contrast, the alleles in NPR, Yawata, and SoOm1 were fixed for the H110R mutation. Consequently, the H110R frequencies were significantly correlated with the LC_{50} values to pyridaben (Fig. S5).

No obvious associations were observed between the LC_{50} values for pyridaben and Log2FC of CYP genes, although *CYP392A3* expression frequently tended to be higher in strains with high H110R frequency in *PSST* (Fig. S4). Linear regression analysis detected significant correlations between the Log2FC and LC_{50} values for five CYP genes (Fig. S6); *CYP385C1* and *CYP388C11* showed negative correlations, while *CYP392A3*, *CYP392B1*, and *CYP392D4* showed positive correlations. The largest slope was 0.950 for *CYP392A3*, although three susceptible strains (Izu2, Yokote, and Iwate) also showed significant increases in *CYP392A3* expression levels (Fig. S4).

In PCA, the first (PC1) and second (PC2) components explained 49.9% of the total variance, and the variances were > 1 in PC1–PC4 (Fig. S7). Plots for PC1 and PC2 indicated higher PC2 values for strains that had high LC_{50} values for pyridaben (NPR, Yawata, SoOm1, and Masu; Fig. 5a). According to factor loading, higher PC2 values suggested higher PSST H110R frequencies and *CYP392A3* expression levels (Fig. 5b). The NPR and Masu strains showed characteristically high expression levels of *CYP392B1*, causing separation from the Yawata and SoOm1 strains on the scatter plot (Fig. 5a). The Izu2 strain was close to the Yawata and SoOm1 strains, likely due to the high expression level of *CYP392A3* in Izu2. This suggested an association of the high PSST H110R frequency and high *CYP392A3* expression level with pyridaben resistance.

3.6. Sequence analysis of CYP392A3

3.6.1. Cloning and sequencing of CYP392A3 alleles of NPR

DNA sequence alignment of the four *CYP392A3* clones of the NPR strain with that of the London strain (*tetur07g06460*; Grbić et al., 2011) showed two insertions with 12 and 9 nucleotides between bases 783 and 784 (c.783_784insGATGCGTTACAA; INS1), and between bases 1,242 and 1,243 (c.1242_1243insAATATTACC; INS2) (Supplementary information 4; INS2+ type in *Section 2.9*). These nucleotide insertions resulted in the insertion of four (p.K261T_V262IinsDALQ; INS1) and three (p.E414_N415insNIT; INS2) amino acid residues, respectively (Fig. S8a; Supplementary information 5). One nonsynonymous SNP (c.314C>A [P105H]) was found in one clone (A3-1; Fig. S8a). We named the variants *Ins-1* and *Ins-2* for P105 and P105H, respectively.

530 *3.6.2. Direct sequencing of CYP392A3 alleles in males of local strains*

531 Partial sequencing of the insertion region revealed that all males of all strains had
532 INS1. However, individuals with INS1, but without INS2 (INS2− type in *Section 2.9*)
533 had two SNPs in their INS1 DNA sequence in comparison with NPR (Table 4). The
534 SNPs were nonsynonymous substitutions, resulting in p.K261_V262insDSLE (Fig.
535 S8b). In the NPS strain, all males (93.8%) had the INS2− type *CYP392A3*, except for
536 one male with the INS2+ type (with both INS1 and INS2), while all NPR, Izu2, and
537 Masu males had the INS2+ type (Table 4). The INS2+ type frequency was ~60% in the
538 SoOm1 and Yawata strains.

539 Amplicon sequencing revealed several nonsynonymous substitutions in INS2−-type
540 alleles. Consequently, the INS2− type included three variants: *Del-1*, *Del-2*, and *Del-3*
541 (Fig. S8b). The *CYP392A3* of males that had INS2 were all *Ins-1* variant.

542 *3.7. Effects of the combination of mutant PSST and highly expressed CYP392A3*

543 *3.7.1. Double introgression of mutant PSST and candidate CYP392A3 alleles (INS2+*
544 *type) into susceptible strain by marker-assisted backcrossing*

545 The LC_{50} values of congenic lines against pyridaben were not significantly different
546 (the 95% CIs overlapped) from their parental lines with the same alleles in *PSST* and
547 *CYP392A3* (Fig. 6a, Table 5).

548 *CYP392A3* expression levels were significantly higher in RR_1 and RR_2
549 compared to SS_1 and SS_2 (two-way ANOVA, $F_{[1,9]} = 86.08$, $P = 6.65 \times 10^{-6}$ and $F_{[1,9]}$
550 $= 103.75$, $P = 3.07 \times 10^{-6}$), respectively, as well as R_1 and R_2 compared to S_1 and
551 S_2 ($F_{[1,9]} = 80.17$, $P = 8.9 \times 10^{-6}$; and $F_{[1,9]} = 20.64$, $P = 0.0014$, respectively; Fig. 6b).
552 Significant differences between reference genes were detected for all lines ($P < 0.01$)

except the combination of R_2 and S_2 ($F_{[1,9]}$ = 3.874, P = 0.081). Two-way ANOVA of Log2FC in *CYP392A3* expression level revealed a significant difference between the R_i and RR_i lines ($F_{[3,19]}$ = 6.666, P = 0.003) while no differences were detected between reference genes ($F_{[1,19]}$ = 0.023, P = 0.88). Multiple comparison analysis indicated that the Log2FCs of RR_1 and RR_2 were not significantly different from R_2 (Tukey–Kramer method, P = 0.545 and 0.74, respectively) but lower than R_1 (P = 0.004 and 0.008, respectively). The difference between R_1 and R_2 was marginal (P = 0.065).

3.7.2. Uncoupling of resistant congenic line (RR_i)-derived PSST (H110R) and CYP392A3 (INS2+ type) alleles

The rates of meiotic recombination between *PSST* and *CYP392A3* loci in F2 males produced by F1 females from SS_1 × RR_1 (n = 79) and SS_2 × RR_2 (n = 75) were 2.5% and 2.7%, respectively (Table S8). Recombinants from SS_2 × RR_2 were one R-INS2− and one H-INS2+, whereas those from SS_1 × RR_1 were two H-INS2+. Consequently, we obtained a series of four lines (R-INS2+, R-INS2−, H-INS2+, and H-INS2−) from the parental crosses of SS_2 and RR_2. However, H-INS2+ did not grow well, so we could obtain only limited data in this line. The following analyses were mainly performed using the R-INS2+, R-INS2−, and H-INS2− strains.

The LC_{50} of H-INS2− (3.55 mg L^{-1}; Fig. 7a, Table 6) was equivalent to or lower than that of S_i and SS_i (Table 5). Nevertheless, the level of *CYP392A3* expression was unexpectedly increased in the H-INS2− line, and was ~9-fold higher than in S_1 (Fig. 7b).

The LC_{50} value of the R-INS2− line was not significantly different from that of the

R-INS2+ line (Fig. 7a, Table 6), and both were significantly lower than those of the R_i and RR_i lines (Table 5). The relative expression levels of *CYP392A3* in the R-INS2+ line were 7.1- or 8.2-fold (with reference to *GAPDH* and *ubiquitin*, respectively) higher than those of S_1, but not significantly different from those of H-INS2− (Fig. 7b). The *CYP392A3* expression level of the R-INS2− line was significantly lower than those of the R-INS2+ and H-INS2− lines, but 2.6-fold higher than S_1 (Fig. 7b). In the H-INS2+ line, the mortality rates were 100% and ≤ 10% at concentrations of > 6 and ≤ 0.6 mg L^{-1}, respectively (Fig. S9a), suggesting high susceptibility. The expression level of *CYP392A3* in H-INS2+ (without replicates) was equivalent to H-INS2− (analyzed simultaneously), and 10-fold higher than S_1 (Fig. S9b).

4. Discussion

A differential maternal effect of pyridaben resistance on the inheritance of resistance in eggs from reciprocal crosses between resistant and susceptible strains was reported previously (Stumpf and Nauen, 2001; Sugimoto and Osakabe, 2014). Maternal effects could be conferred by a target site mutation encoded in mitochondrial DNA, as reported for bifenazate resistance caused by mutations in mitochondrial cytochrome *b* (Van Leeuwen et al., 2006; Van Nieuwenhuyse et al., 2009). However, the maternal effect of pyridaben-resistance is limited to eggs, and is weakened or absent in the adult stage (Devine et al., 2001; Stumpf and Nauen, 2001; Sugimoto and Osakabe, 2014; Van Pottelberge et al., 2009). This makes the involvement of a mitochondrial DNA-encoded target site mutation less likely. Indeed, there was no evidence of contributions of nonsynonymous mutations in the mitochondria encoded subunits ND1 and ND5 from complex I in NPR.

599 Bajda et al. (2017) suggested that maternally synthesized PSST proteins (H110 or
600 H110R) may be important for determination of susceptibility in early life stages of eggs.
601 In contrast, we found higher CYP activity in the pyridaben-resistant strain compared to
602 the susceptible strain at the egg stage, and the maternal effect in eggs was correlated
603 with CYP activity. Moreover, CYP inhibition treatment of maternal females of the
604 resistant strain markedly reduced the resistance levels of their eggs to pyridaben. Eggs
605 inherit mitochondria from females; thus, PSST protein of eggs, at least in the early
606 stages, is likely produced by the mother (Bajda et al., 2017). Our results suggested
607 additional involvement of *CYP*s with the egg limited maternal effects. This also
608 supports the importance of *CYP*s in pyridaben resistance (Kim et al., 2006; Sugimoto
609 and Osakabe, 2014; Van Pottelberge et al., 2009), in combination with mutant *PSST*.
610 However, several susceptible strains exhibited high expression levels of several *CYP*
611 genes, including *CYP392A3*, a candidate factor causing high resistance levels in this
612 study, without *PSST* H110R mutation. This suggest that *CYP*s alone might only
613 marginally contribute to resistance, and other mechanisms, such as cumulative or
614 synergistic effects, are involved in high resistance levels.
615 PCA following RT-qPCR analysis indicated the effects of the combination of
616 mutant *PSST* and high expression of *CYP392A3* on pyridaben resistance. *CYP392A3*
617 was one of the *CYP*s composing a cluster closely linked to *PSST* and located within the
618 QTL region, as reported by Bajda et al. (2017). To validate the combined effect, we
619 confirmed that double introgression of resistant strain-derived mutant *PSST* and INS2+
620 type *CYP392A3* (alternative marker for overexpression) alleles conferred high
621 resistance levels (LC_{50} > 10,000 mg L^{-1}) in a different susceptible genetic background.
622 The highly resistant congenic lines (RR_1 and RR_2) retained higher *CYP392A3*

623 expression levels, although they were lower than one of the two parental resistant
624 strains, R_1. The resistance levels of the congenic resistant lines (RR_1 and RR_2)
625 were conspicuously higher than the previous congenic lines produced by introgression
626 of mutant PSST (LC_{50} = ~200 mg L^{-1}), as described by Bajda et al. (2017). The
627 susceptible strain used in this study was the same strain used by Bajda et al. (2017).
628 Therefore, the congenic strains obtained in these studies had a similar genetic
629 background. However, the donor of mutant PSST in the study of Bajda et al. (2017)
630 (MR-VP) was a multi-resistant Belgium strain, which showed no overexpression of
631 *CYP392A3* or *CYP*s in the same cluster (Khalighi et al. 2016). This suggested that the
632 high pyridaben resistance levels detected in this study were likely conferred by the
633 combination of mutant *PSST* and overexpressed *CYP392A3*, or by other unknown
634 factors linked to *CYP392A3*.
635 To determine the effects of overexpressed *CYP392A3* combined with mutant *PSST*,
636 we attempted to uncouple these alleles derived from the resistant congenic strains.
637 However, of the four resulting uncoupled lines, the *CYP392A3* expression levels of H-
638 INS2− and R-INS2− lines were unexpectedly high and intermediate between resistant
639 and susceptible parental congenic lines, respectively. This suggested that separation of
640 the alternative marker (indel) and an actual determinant of *CYP392A3* expression level
641 occurred during the uncoupling process between closely linked *PSST* and *CYP392A3*
642 loci, e.g., meiosis in the ovary of F1F to produce F2M eggs. Therefore, we could not
643 evaluate the effects of mutant PSST alone in this study. Nevertheless, together with the
644 observation that the H-INS2+ line with high *CYP392A3* expression level was
645 undoubtedly susceptible, this clearly indicated that overexpression of *CYP392A3* alone
646 did not confer pyridaben resistance. Moreover, this was also compatible with the RT-

qPCR data of the local populations; significant increases in *CYP392A3* expression were detected not only in resistant strains, but also in the three susceptible strains.

The LC_{50} value of R-INS2− equivalent to that of R-INS2+ showed that the indel mutation of *CYP392A3* was not functionally involved in pyridaben resistance. On the other hand, the expression level of the R-INS2− line was significantly lower than the R-INS2+ line. This made it difficult to interpret the combined effects of *CYP392A3* expression levels and mutant *PSST* on pyridaben resistance levels. Therefore, we tentatively tested the effects of CYP inhibition by PBO on the resistance levels of the uncoupled congenic lines and their parental lines (Supplementary information 6). Consequently, 100% of R-INS2+, R-INS2−, R_2, and RR_2 females pretreated with PBO died after spraying with pyridaben at a concentration of > 16 mg L^{-1}, equivalent to the susceptibility of parental susceptible lines (S_i and SS_i) without PBO treatment shown in Fig. 6a. All PBO-pretreated females died at doses > 0.16 mg L^{-1} in S_2 and SS_2, whereas slightly higher pyridaben concentration of > 0.8 mg L^{-1} was required to kill PBO-pretreated females of H-INS2− with a high *CYP392A3* expression level. This also suggested a contribution of *CYP392A3* or other *CYP*s to pyridaben resistance in NPR, where the presence of mutant PSST was essential.

Little information is available regarding how the target site mutations that, in isolation, provide only low resistance, contribute to high resistance levels. We tentatively analyzed the correlations between LC_{50} values and Log2FCs in *CYP392A3*, in congenic and parental lines with mutant PSST (R_i, RR_i, R-INS2+, and R-INS2−). The Log2FC values of *CYP392A3* were recomputed using S_1 as a common calibrator for standardization prior to the analysis. The results indicated a significant positive correlation between these factors (Fig. S10). Overall, *CYP392A3* still remains a

candidate gene for the high pyridaben resistance level, due to the possibility of synergistic effects.

Our findings clearly show the contribution of CYP to the high resistance levels of pyridaben. Indeed, total activity of CYPs tended to be correlated with pyridaben resistance, especially in eggs, although the trend was marginal in adult females. A spurious correlation of *CYP392A3* should also be considered, however, because the locus of this gene is closely linked to PSST and formed a cluster including the highly similar *CYP392A1* and *CYP392A4* (identity > 84%). Moreover, genome wide QTL analysis using SNPs in MR-VP suggested the involvement of a cytochrome P450-reductase gene in pyridaben resistance in *T. urticae* (Snoeck et al. 2019). As CYP contributes in ways other than increasing expression of a specific, single gene (e.g., functional changes and cumulative effects of multiple CYPs), there may be contributions of *trans*-acting elements genetically linked to *PSST*, and *CYP392A3* selected as a candidate resistance factor in this study.

5. Conclusion

Our findings clearly indicated that the high pyridaben resistance levels were conferred by the PSST H110R mutation and CYP392A3, or by genetically associated but unknown factors. Due to the clear response to the inhibition of CYP activity, the unknown factors should be associated with *CYP* genes via *cis*- or *trans*-acting regulation. We concluded that the high pyridaben resistance is probably due to a synergistic or cumulative effect of the combination of mutant PSST and associated CYP activity.

Accession numbers

Raw sequencing data for mitochondrial genomic DNA were submitted to the NCBI database (BioProject PRJNA663742). Raw RNA-seq data were deposited to DDBJ under accession number DRA010496. The cDNA and genomic DNA sequences of *CYP392A3* variants are available from the DDBJ/EMBL/GenBank databases under accession numbers LC581389–LC581418.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at ################.

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889
890

891 **Figure legends**

892

893 **Fig. 1.** Pyridaben concentration–mortality plots for F1 eggs of NPS♀ × NPS♂ (open
894 triangles), NPS♀ × NPR♂ (gray triangles), NPR♀ × NPS♂ (gray circles), and NPR♀ ×
895 NPR♂ (solid circles).

896

897 **Fig. 2.** 7EFC-*O*-deethylation activity (a) in adult females and eggs of pyridaben-
898 resistant (NPR) and -susceptible (NPS) strains, and (b) in F1 generation after reciprocal
899 crosses. RS: NPR♀ × NPS♂, SR: NPS♀ × NPR♂. *P*-values were computed by one-way
900 ANOVA, assuming that the replicates were paired observations.

901

902 **Fig. 3.** Synergistic effects of PBO-treated females on the egg hatchability of the
903 pyridaben-resistant strain (NPR) after pyridaben treatment. The symbols "+" and "–"
904 represent treated and untreated, respectively. Error bars represent the standard error.
905 Different letters above bars indicate significant differences in egg hatchability ($P <$
906 0.05) by the Tukey–Kramer method.

907

908 **Fig. 4.** (a) Coverage of contigs, (b) averaged ($\log_2$) expression level (A-values), and (c)
909 $\log_2$ fold changes (M-values) for differentially expressed genes (DEGs) of cytochrome
910 P450 (CYP) upregulated in NPR in comparison with NPS, in adult females induced
911 with pyridaben (open circles) and non-induced adult females (open triangles). Bars
912 indicate average values between the induced and non-induced samples. A-value:
913 averaged expression level ($\log_2$) between NPR and NPS; M-value: $\log_2$-fold change in

914 NPR. *CYP389C1* was automatically divided into two distinctive contigs and parameters
915 were computed separately.
916
917 **Fig. 5.** Principal component analysis of the frequency of PSST H110R mutation and
918 standardized log2-fold change (−ΔΔCq) of expression levels in 12 CYP genes analyzed
919 in 11 *T. urticae* strains. (a) Scatter plot of the first (PC1) and second (PC2) principal
920 components. (b) Factor loadings. Solid plots in (a) show strains resistant to pyridaben
921 (LC_{50} > 200 mg L^{-1}).
922
923 **Fig. 6.** Pyridaben susceptibility and CYP392A3 expression levels in parental and
924 congenic lines after double introgression. (a) Concentration–mortality plots for
925 backcrossed lines (RR_i and SS_i; i = 1 or 2) and their parental single female lines (R_i
926 and S_i). RR_1 (solid rhombuses) and SS_1 (solid squares) were separated from the
927 same backcrossed line derived from the parental cross between an S_1 female (open
928 squares) and an R_1 male (open rhombuses). RR_2 (solid circles) and SS_2 (solid
929 triangles) were derived from the cross between an S_2 female (open triangles) and R_2
930 male (open circles). (b) $\log_2$ fold change (Log2FC = −ΔΔCq)) in the expression level of
931 *CYP392A3* of R_i and RR_i lines against S_i and SS_i lines, respectively. Gray and
932 open bars represent Log2FC calculated using *GAPDH* and *ubiquitin* as reference genes,
933 respectively. Asterisks above bars indicate that *CYP392A3* expression levels evaluated
934 with ΔCq values are significantly higher in R_i and RR_i in comparison with S_i and
935 SS_i, respectively (two-way ANOVA, *** $P < 0.001$, ** $P < 0.01$). Significant
936 differences between reference genes were detected for all genes tested ($P < 0.01$) except
937 in the combination of R_2 and S_2 ($P = 0.081$). Different letters above bars indicate

938 significant differences of log2FC values among lines (Tukey–Kramer method, $P <$
939 0.05).
940
941 **Fig. 7.** Resistance levels and *CYP392A3* expression levels of strains, with uncoupling of
942 *PSST* and *CYP392A3* from the pyridaben-resistant strain. (a) Concentration–mortality
943 plots for congenic lines, with uncoupling of *PSST* and *CYP392A3* alleles of RR_2. R-
944 INS2+: *PSST* H110R + *CYP392A3* with insertion (originally found in resistant strains),
945 R-INS2−: *PSST* H110R + *CYP392A3* with deletion (originally found in susceptible
946 strains), H-INS2−: *PSST* H110 + *CYP392A3* with deletion. (b) The $\log_2$ fold change
947 (Log2FC = $-\Delta\Delta$Cq) in expression level of *CYP392A3* of R_i and RR_i lines against
948 S_1 line. Gray and open bars represent Log2FC calculated using *GAPDH* and *ubiquitin*
949 as reference genes, respectively. Different letters above bars indicate significant
950 differences in log2FC values among lines (Tukey–Kramer method, $P < 0.05$).
951

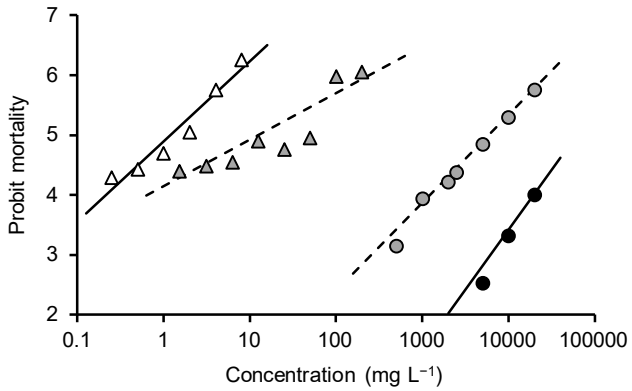

Probit mortality
7
6
5
4
3
2
0.1
1
10
100
1000
10000
100000
Concentration (mg L⁻¹)

Fig. 1.

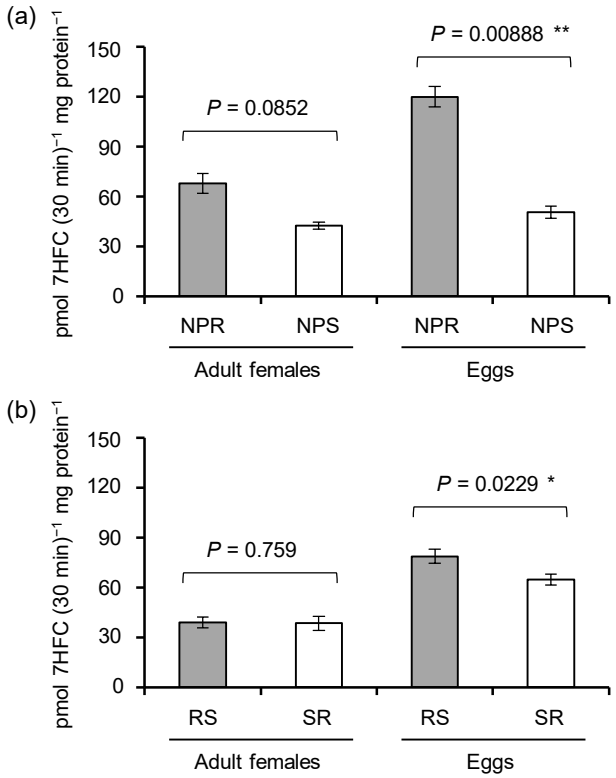

(a)
pmol 7HFC (30 min)⁻¹ mg protein⁻¹
150
120
90
60
30
0
P = 0.0852
P = 0.00888 **
NPR
NPS
NPR
NPS
Adult females
Eggs
(b)
pmol 7HFC (30 min)⁻¹ mg protein⁻¹
150
120
90
60
30
0
P = 0.759
P = 0.0229 *
RS
SR
RS
SR
Adult females
Eggs

Fig. 2.

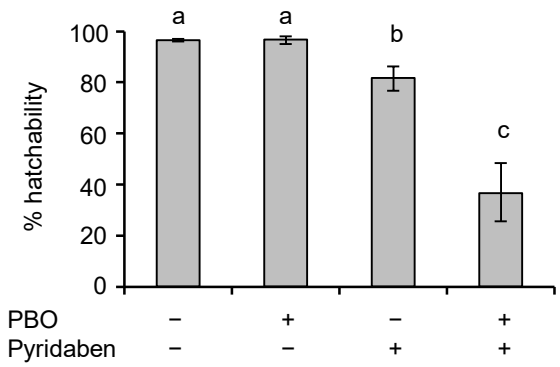


Fig. 3.

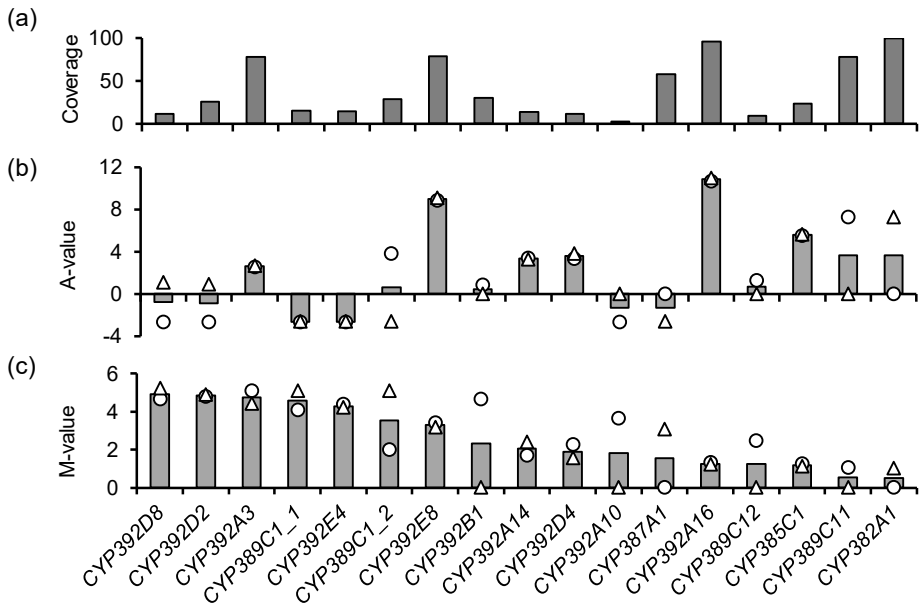

(a)
Coverage
100
50
0
(b)
A-value
12
8
4
0
-4
(c)
M-value
6
4
2
0
CYP392D8
CYP392D2
CYP392A3
CYP389C1_1
CYP392E4
CYP389C1_2
CYP392E8
CYP392B1
CYP392A14
CYP392D4
CYP392A10
CYP387A1
CYP392A16
CYP389C12
CYP385C1
CYP389C11
CYP382A1

Fig. 4.

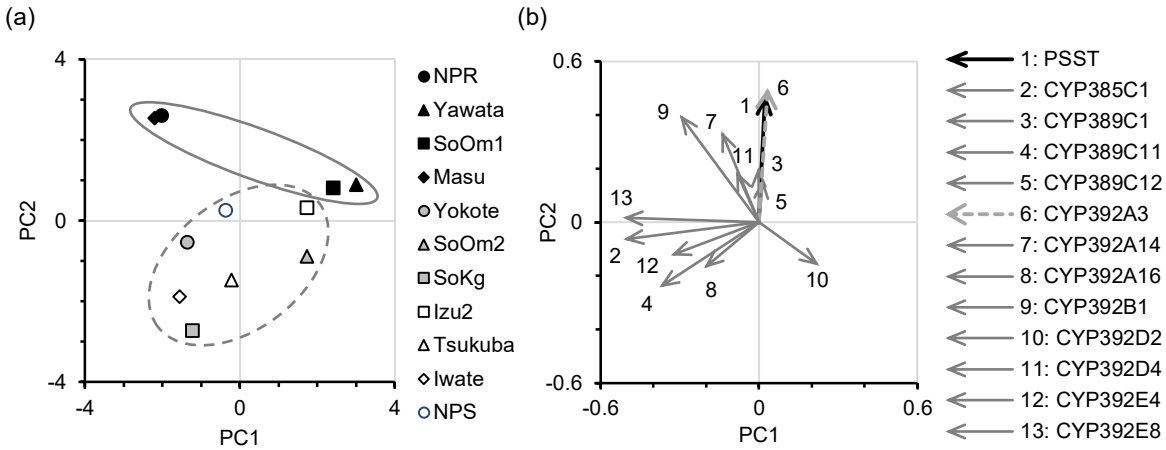

(a)
(b)
PC1
PC2
NPR
Yawata
SoOm1
Masu
Yokote
SoOm2
SoKg
Izu2
Tsukuba
Iwate
NPS
1: PSST
2: CYP385C1
3: CYP389C1
4: CYP389C11
5: CYP389C12
6: CYP392A3
7: CYP392A14
8: CYP392A16
9: CYP392B1
10: CYP392D2
11: CYP392D4
12: CYP392E4
13: CYP392E8

Fig. 5

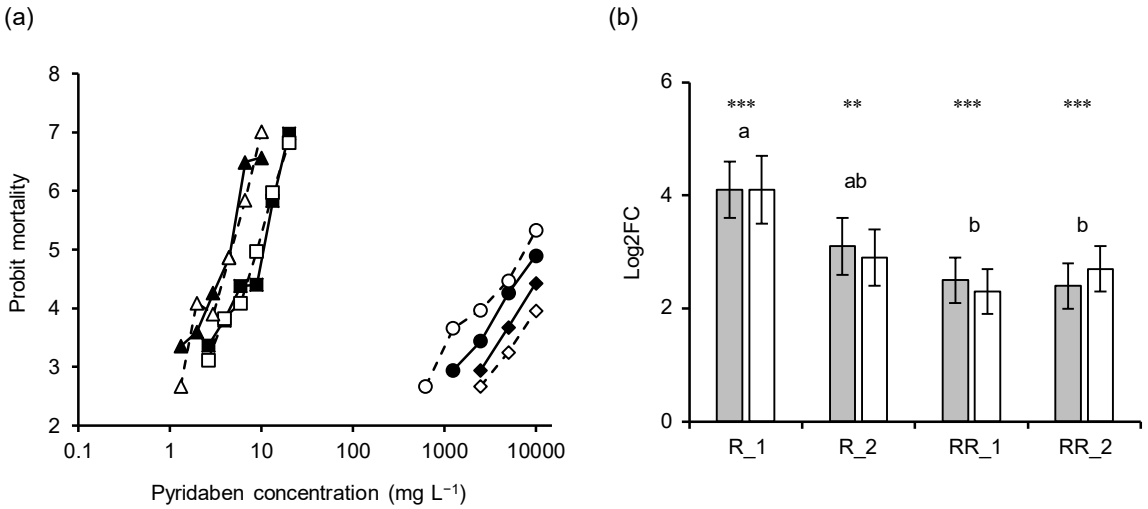

(a)
Probit mortality
8
7
6
5
4
3
2
0.1
1
10
100
1000
10000
Pyridaben concentration (mg L⁻¹)
(b)
Log2FC
6
4
2
0
***
**
***
***
a
ab
b
b
R_1
R_2
RR_1
RR_2

Fig. 6.

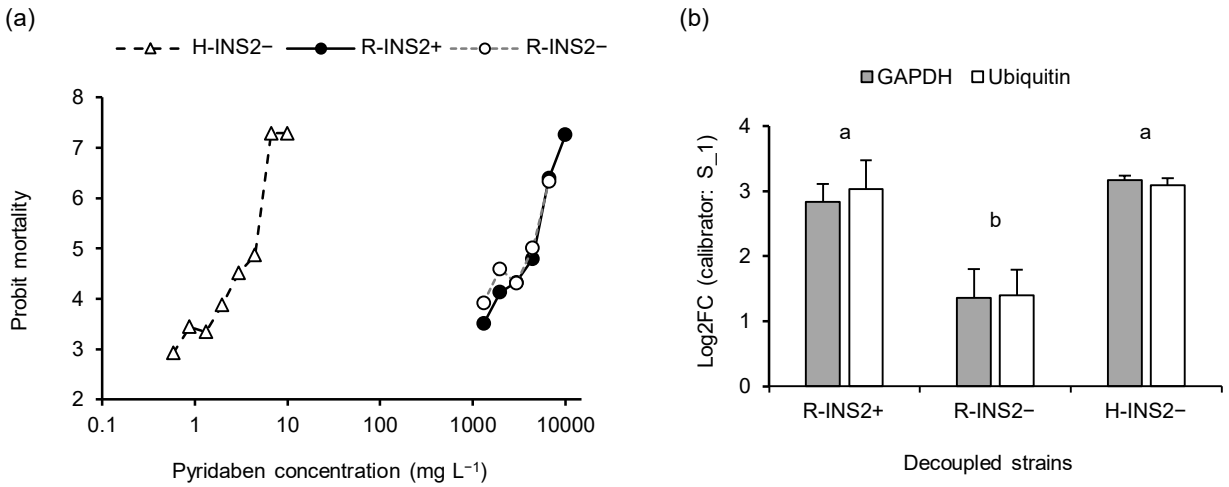

(a)
H-INS2−
R-INS2+
R-INS2−
Probit mortality
8
7
6
5
4
3
2
0.1
1
10
100
1000
10000
Pyridaben concentration (mg L−1)
(b)
GAPDH
Ubiquitin
Log2FC (calibrator: S_1)
4
3
2
1
0
a
b
a
R-INS2+
R-INS2−
H-INS2−
Decoupled strains

Fig. 7.

Table 1. Collection records for *Tetranychus urticae* strains.

Strains	Dates	Localities	Host plants
NPR [a]	May, 2010	Heguri, Nara Pref. (34.6°N, 135.7°E)	Rose
NPS [a]	1998	Katsuragi, Nara Prefecture (34.5°N, 135.7°E)	Chrysanthemum
Iwate [b]	Oct, 1999	Morioka, Iwate Pref. (39.7°N, 141.2°E)	Apple
Yokote [b]	Jun, 2014	Yokote, Akita Pref. (39.3°N, 140.6°E)	Apple
Izu2 [b]	Feb, 2013	Izunokuni, Shizuoka Pref. (35.0°N, 138.9°E)	Strawberry
Masu [b]	Jan, 2012	Shizuoka (35.0°'N, 138.5°E)	Strawberry
Yawata [b]	Oct, 2014	Yawata, Kyoto Pref. (34.9°N, 135.7°E)	Japanese pear
SoKg [c]	Jan, 2012	Shizuoka (34.8°N, 138.0°E)	Strawberry
SoOm1 [b]	Jan, 2012	Omaezaki, Shizuoka Pref. (34.6°N, 138.1°E)	Strawberry
SoOm2	Apr, 2013	Omaezaki, Shizuoka Pref. (34.6°N, 138.1°E)	Strawberry
Tsukuba [b]	Unknown	Laboratory strain	Kidney bean

[a] Sugimoto and Osakabe (2014) and Bajda et al. (2017)

[b] Osakabe et al. (2017)

[c] Sugimoto et al. (2020)

Table 2. Pyridaben susceptibility of F1 eggs from reciprocal crosses between resistant NPR and susceptible NPS strains

Crosses (♀ × ♂)	n ± SD [a]	LC_{50} (mg L^{-1})	95% confidence limit	Regression lines	RR [b]
NPS × NPS	819 ± 69.6	1.19	1.11–1.27	$Y = 1.34\,X + 4.90$	1
NPS × NPR	534.4 ± 41.4	12.6	11.15–14.18	$Y = 0.78\,X + 4.15$	10.59
NPR × NPS	852 ± 179.4	5916	5593–6268	$Y = 1.47\,X - 0.56$	4971
NPR × NPR	457.3 ± 22.5	61,310	44,956–100,599	$Y = 2.00\,X - 4.58$	51,521

[a] Averaged number of eggs used for the test at each concentration ± standard deviation (SD)

[b] Resistance ratio

Table 3. Logarithmic dose–probit mortality regression line data against pyridaben expressed as LC_{50}, slope, and resistance ratio (RR) in laboratory-selected resistant (NPR) and susceptive (NPS) strains and field strains without laboratory selection.

Strains	LC_{50} (mg L^{-1})	95% CI (mg L^{-1})	Regression line	DF [a]	RR [b]
NPR	7756	6061–10915	$Y = 1.64\,X - 1.36$	1	1925
NPS	4.03	3.53–4.60	$Y = 2.06\,X + 3.75$	4	1
Iwate	1.76	1.59–1.94	$Y = 3.51\,X + 4.14$	4	0.44
Yokote	11.5	9.02–14.5	$Y = 1.21\,X + 3.71$	7	2.85
Izu2	8.04	7.16–9.03	$Y = 2.80\,X + 2.47$	4	2.00
Masu	272.2	193.3–379.0	$Y = 0.71\,X + 3.27$	5	67.54
Yawata	>10,000				>2481
SoKg	11.1	8.82–13.8	$Y = 1.07\,X + 3.88$	7	2.75
SoOm1	>10,000				>2481
SoOm2	11.1	9.81–12.8	$Y = 2.15\,X + 2.74$	5	2.75
Tsukuba	5.96	5.34–6.67	$Y = 2.71\,X + 2.90$	4	1.48

[a] Degree of freedom for regression line

[b] Resistance ratio to NPS

Table 4. Number of males of the two indel types (INS2+/INS2−) in CYP392A3 detected by the direct sequencing

Strains	No of males tested	INS2− type (%) [a]	INS2+ type (%) [b]
NPS	16	15 (93.8)	1 (6.3)
Izu2	12	0 (0)	12 (100)
Masu	12	0 (0)	12 (100)
SoOm1	10	4 (40)	6 (60)
Yawata	11	4 (36.4)	7 (63.6)
NPR	8	0 (0)	8 (100)

[a] c.783_784insGATTCGTTAGAA without c.1242_1243ins

[b] c.783_784insGATGCGTTACAA with c.1242_1243insAATATTACC

Table 5. Logarithmic dose–probit mortality regression line data against pyridaben expressed as LC_{50}, slope, and resistance ratio (RR) in backcrossed lines (RR_i and SS_i) and their parental single female lines (R_i and S_i).

Lines [a]	LC_{50} (mg L^{-1})	95% CI (mg L^{-1})	Regression line	DF [b]	RR [c]
RR_1	17,048	12620–31510	$Y = 2.47\,X - 5.44$	1	4230
SS_1	8.95	8.28–9.70	$Y = 3.76\,X + 1.42$	4	2.22
RR_2	10,989	8943–14870	$Y = 2.27\,X - 4.18$	2	2727
SS_2	4.13	3.84–4.43	$Y = 4.29\,X + 2.36$	4	1.02
R_1	29,592	17367–157164	$Y = 2.22\,X - 4.92$	1	7343
S_1	8.39	7.82–9.03	$Y = 4.26\,X + 1.06$	4	2.08
R_2	7711	6398–9808	$Y = 1.98\,X - 2.69$	3	1913
S_2	4.32	4.02–4.65	$Y = 4.20\,X + 2.33$	4	1.07

[a] RR_1 and SS_1 lines were separated from the same backcrossed line started from the parental cross between an S_1 female and an R_1 male. RR_2 and SS_2 lines were derived from the cross between an S_2 female and an R_2 male.

[b] Degree of freedom for regression line

[c] Resistance ratio to NPS in Table 3

Table 6. Logarithmic dose–probit mortality regression line data against pyridaben expressed as LC_{50}, slope, and resistance ratio (RR) in uncoupled lines.

Lines[a]	LC_{50} (mg L^{-1})	95% CI (mg L^{-1})	Regression line	DF [b]	RR [c]
R-INS2+	3820	3531–4136	$Y = 3.91X - 8.99$	4	947.9
R-INS2−	3482	3148–3883	$Y = 2.74X - 4.72$	3	864.0
H-INS2−	3.55	3.29–3.84	$Y = 3.62X + 3.01$	6	0.88

[a] Uncoupled lines were established from a parental cross between SS_2 female and RR_2 male.

[b] Degree of freedom for regression line

[c] Resistance ratio to NPS in Table 3

Supplementary information 1:

Quantitative real-time PCR (qPCR) condition

Section *2.7.2. PSST mutation frequency analysis*

The qPCR was performed using TB Green Premix Ex TaqTM II (Tli RNaseH Plus) (TaKaRa Bio, Kusatsu, Japan) for 20 μL reaction solution containing 2 ng DNA samples and forward and reverse primers (0.4 μM each). The reaction mixtures were incubated at 95°C for 10 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s using the LightCycler 96 System (Roche Diagnostics, Tokyo, Japan).

Section *2.7.3*, and *2.9. RT-qPCR analysis for CYPs up-regulated in RNA-seq*

The gene expression levels of the 12 CYP genes were evaluated for the cDNA samples by RT-qPCR analysis using Lightcycler 96 system (Roche Diagnostics). The reaction mixture (20 μL) consisted of 10 μL of TB Green Premix Ex Taq II (Tli RNaseH Plus), cDNA synthesized from 1–2 ng total RNA, and a primer set (0.4 μM each; Table S2). The PCR conditions were pre-incubation at 95°C for 5 s; followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and 72°C for 30 s.

Supplementary information 2:

Sanger sequencing

Section 2.8.1. *PCR amplification of cDNA for cloning and sequencing of CYP392A3 in NPR*

PCR amplification of cDNA: The *CYP392A3* were amplified by PCR in a reaction mixture (20 μL) consisted of cDNA synthesized from 1–2 ng total RNA, 0.4 unit KOD FX Neo, 1×KOD FX Neo Buffer, 0.4 mM dNTPs, and 0.25 μM forward and reverse primers. PCR amplification was performed with temperature cycles at 94°C for 2 min; followed by 40 cycles of 98°C for 10 sec, 60°C for 30 sec, and 68°C for 45 sec; and at 68°C for 7 min.

Cycle sequencing reaction: Cycle sequencing reaction was performed using BigDye Terminator v3.1 Cycle sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA). Reaction solution (20 μL) was composed of 136–160 ng plasmid DNA, 3.5 μL 5×Sequencing Buffer, 1 μL BigDye Terminator v3.1 Ready Reaction Mix, and 0.075 μM primer. The solution was reacted with temperature cycles at 96°C for 1 min; followed by 25 cycles of 96°C for 10 s, 50°C for 30 s, and 60°C for 4 min. For purification of the reaction solution, 2.5 μL EDTA (0.125 M, pH 8.0) and 30 μL ethanol (99.5%) were added and mixed. After 15 min, the reaction was centrifuged (15,000 rpm) at 4°C for 30 min. The supernatant was discarded by pipetting. After 70% ethanol wash, the pellet was dried and dissolved in 10–20 μL of Hi-Di Formamide (Thermo Fisher Scientific). Then, applied for the analysis by the Genetic Analyzer 3130 (Thermo Fisher Scientific).

Sections *2.7.2*, *2.8.2*, and *2.9.* *Direct sequencing*

Crude DNA sample preparation: Adult females and males were individually homogenized in 20 μL lysis buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA [pH 8.0], 0.5% IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO, USA), 10 mM NaCl, and 0.5 μL proteinase K [350 U mL−1; TaKaRa Bio]). The homogenate was kept at 65°C for 15 min and then heated at 95°C for 10 min using a thermal cycler (TP3000, TaKaRa Bio). The crude DNA samples of females and males were diluted 40- and 10-fold with 0.1×TE buffer, respectively, before used for PCR amplification.

Template amplification: The *CYP392A3* and *PSST* were amplified by PCR in a reaction mixture (5 μL) consisted of 1 μL 40-fold diluted crude DNA solution, 0.1 unit KOD FX Neo, 1×KOD FX Neo Buffer, 0.4 mM dNTPs, and 0.25 μM forward and reverse primers for *CYP392A3* and 0.5 μM forward and reverse primers for *PSST*. PCR amplification was performed with temperature cycles at 94°C for 2 min; followed by 40 cycles of 98°C for 10 sec, 60°C for 30 sec, and 68°C for 90 sec; and at 68°C for 2 min.

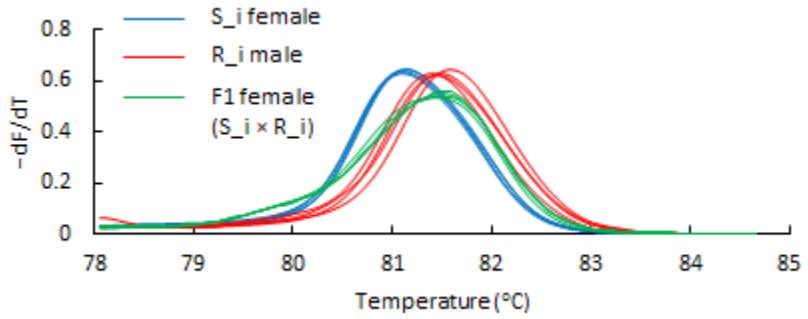
Purification of PCR products: For purification, 20 μL of 12.5% polyethylene glycol (PEG8000, Promega)–12.5M $MgCl_2$ solution was mixed with the PCR solution (5 μL). The mixture was centrifuged by 15, 000 rpm, at 4°C, for 30 min. After washing with 70% ethanol twice, the pellet was dissolved in 20 μL Milli-Q water. The purified PCR products were used for cycle sequencing.

Cycle sequencing reaction: Cycle sequencing reaction was performed using BigDye Terminator v3.1 Cycle sequencing Kit. Reaction solution (5 μL) was composed of 0.875 μL 5×Sequencing Buffer, 0.25 μL BigDye Terminator v3.1 Ready Reaction Mix, and 0.075 μM primer. The solution was reacted with temperature cycles at 96°C for 1 min; followed by 25 cycles of 96°C for 10 s, 50°C for 30 s, and 60°C for 4 min. For purification of the reaction solution, 1.25 μL EDTA (0.125 M, pH 8.0) and 15 μL ethanol (99.5%) were added and mixed. After 15 min, the reaction was centrifuged (15,000 rpm) at 4°C for 30 min. The supernatant was discarded by pipetting. After 70% ethanol wash, the pellet was dried and dissolved in 10–20 μL of Hi-Di Formamide (Thermo Fisher Scientific). Then, applied for the analysis by the Genetic Analyzer 3130 (Thermo Fisher Scientific).

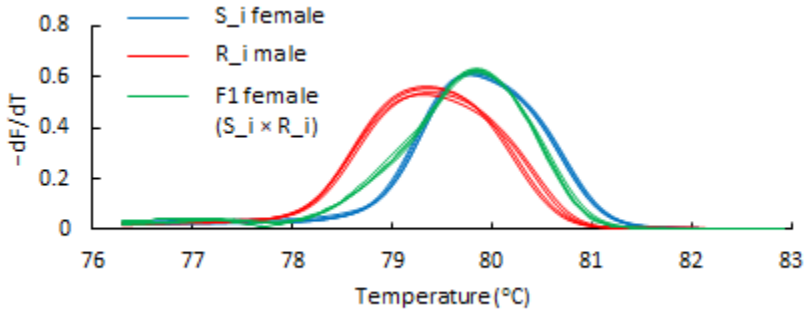
Section *2.9.2* *High resolution melting analysis*

Reaction solution (10 μL) was composed of 5 μL of TB Green Premix Ex Taq II (Tli RNaseH Plus), 0.4 μL each of 10 μM forward and reverse primers, 2.2 μL of distilled water, and 2 μL of crude DNA sample. Sequences of the forward and reverse primers for PSST were 5′-ACGTGGTTCATTATGGCCTTTG-3′ and 5′-CATCAGCTTGCCTAGGACTTG-3′, respectively, and for CYP392A3 were 5′-CTAACCTGGGCCAGCATGTAC-3′ and 5′-ATACGCTGGACTTCATAAAGAAAGG-3′, respectively. The PCR conditions consisted of preheating for 5 s at 95°C, followed

by 40 cycles of 5 s at 95°C, 30 s at 60°C, and 30 s at 72°C and one cycle of 60 s at 95°C, 60 s at 40°C, and 1 s at 65°C. HRM was then performed by heating to 97°C with 15 readings per 1°C.



Shapes of melting curve of PSST fragments for four pairs of parental S_i (H110/H110) females and R_i (H110R) males and their F1 females (H110/H110R; one F1 female for each pair)



Shapes of melting curve of CYP392A3 fragments for four pairs of parental S_i (INS2−/INS2−) females and R_i (INS2+) males and their F1 females (INS2−/INS2+; one F1 female for each pair)

Supplementary information 3:

Alignment of NAD1 and NAD5 between the pyridaben susceptible (NPS) and pyridaben resistant (NPR) strain of T. urticae

The position of the non-synonymous sequence polymorphism (resulting in M495L) in *NAD5* of NPS is indicated in red font.

NAD5

```
          10         20         30         40         50         60         70
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  ATTATTTTTATTAATATATTTAAAATTTTTTTGATAACGTCTTTAATTATATCTTTAATAATGATTAATA
NPR  ............................................................................

          80         90        100        110        120        130        140
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  ATACTTTTTTAATAAATTTTCAAATATTAATTATTTTAAACGAAATATTTTTAAACCTTTATAAAAATTT
NPR  ............................................................................

          150        160        170        180        190        200        210
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TATATTAGTGTTAGACATATATTCATTTTCTTTTTTCTTTATTGTATCAATTGTTGTTTTTAATGTATTA
NPR  ............................................................................

          220        230        240        250        260        270        280
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  AGATTCATAAATATTTATATAAATTCTAACAAAAATATAAAAGTTTTTTTTTTTTATAACAAAAATTTTTA
NPR  ............................................................................

          290        300        310        320        330        340        350
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TTATTTCTATATTTTTACTTGTATTTTCATTTAATTTATGAACAATAATTTTAGGTTGGGAAGGTTTAGG
NPR  ............................................................................

          360        370        380        390        400        410        420
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  AATAAGATCTTTTTTTTTAATTTTTTATTACAACAATTTTGAGAGATGAAAAAGAGCAATTAAAACATTT
NPR  ............................................................................
```

```
          430        440        450        460        470        480        490
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  ATTAATAATAAAATAGGGGATTGTTTAATTTTAATATCTATAATTTACTCAACTATAAATTTAAATTCAT
NPR  ............................................................

          500        510        520        530        540        550        560
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TTAAAATAGTTACATTAATATTCTTAATTTCAATAATAACAAAAAGTGCACAATATCCATTTATATCATG
NPR  ............................................................

          570        580        590        600        610        620        630
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  GTTACCTATAGCTATAGCCGCCCCCACTCCAATTTCAGCAATAGTTCATAGTTCAACTCTTGTAACAGCA
NPR  ............................................................

          640        650        660        670        680        690        700
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  GGGTTATTTATTATATTTCGTTTAATTAATAATTTTTTTACAAAAACAAATTCAAATCTAATTGTCAATT
NPR  ............................................................

          710        720        730        740        750        760        770
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TATGCTTATTATCAATATTTGTTAGAGGTATAAAAGCTGTAAGAGAAAAAGATATAAAGAAAATAATTGC
NPR  ............................................................

          780        790        800        810        820        830        840
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TTTATCAACATTAAGACAAATTGGATTAATTTTTTTTTTTTTAATTAATAACATAAAAATTATTGCATTT
NPR  ............................................................

          850        860        870        880        890        900        910
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  ATTTATATATGTAATCATGCTTTATTTAAATCTCTAATTTTTATTAATATAGGTTTTATAATGATAAATA
NPR  ............................................................

          920        930        940        950        960        970        980
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  ACTTTTCAAATCAATTGAAATTCAATATATTTAATAAAAATATGTGTTCAATCTTTATTTTATCATATAA
NPR  ............................................................

          990        1000       1010       1020       1030       1040       1050
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  AATTTCTTGTTTAAACTTAATAAATTTGTCTTTTTTTTCATCTTTTTTTATTAAAGAAAAAATATTAATA
NPR  ............................................................
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```
          1060       1070       1080       1090       1100       1110       1120
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  AATTTAAGATCAAATTTTTTTAGTATAATAAAATTCATCATTTTTTTAGTAAGAAGCTTCTTTACTATAA
NPR  ............................................................................

          1130       1140       1150       1160       1170       1180       1190
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  ACTATAGAATTAAAATAATATTCTTTTTTAATAGAAAAAACTTTAAAATTAAAAGAAATAATGAATTTAA
NPR  ............................................................................

          1200       1210       1220       1230       1240       1250       1260
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  AACAAAAAATTATTATAAATCACTTTTTTTAATAAATATCTTTTCAATTTGTTATAGAAGATTCATAATA
NPR  ............................................................................

          1270       1280       1290       1300       1310       1320       1330
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  GAAATAATTTTATTTGTTGATATAATAAATCTATTAATTATAATATTTTATTTAATTTCAATAACAATAA
NPR  ............................................................................

          1340       1350       1360       1370       1380       1390       1400
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  ATTACAAAATTTATTCAATAAGTTTTAGTTTTATTAATTCTTTAGCTTATACAGAATTTAGTATATATAT
NPR  ............................................................................

          1410       1420       1430       1440       1450       1460       1470
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TTATCCTTTTAAAATAATAAAATATAGTTTAGATATTAATGAGTTATGGATAGAAAAGATTTCTCTAAAC
NPR  ............................................................................

          1480       1490       1500       1510       1520       1530       1540
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TTTTATTTTTTATTAAAAAATAAAATTATTTTTAAAACTTTCAACATAAATTTTAATTTAATTATAATAT
NPR  ............A...........................................................

          1550       1560
     ....|....|....|....|....|.
NPS  TATTAATTATTTTAATATTTATTTAA
NPR  .........................
```

NAD1

```
          10        20        30        40        50        60        70
     ....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  ATTTTTATTTTTGAATTTTTATTAATACTTTTTTTACTTATAAGAATTATATTTATCACTTTAATAGAAC
NPR  ............................................................

          80        90       100       110       120       130       140
     ....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  GAAAAGTTTTAGGAGTTCTTAATAATCGAAAGAGACCTAACTTTTTTTTTATAAATAGTTTTCTTCAATC
NPR  ............................................................

          150       160       170       180       190       200       210
     ....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TTTAATAGATTTTATCAAATTATTAACAAAAAAAGTTTTAAAATTAAATTTTATTTTAAAAAATTTTTGA
NPR  ............................................................

          220       230       240       250       260       270       280
     ....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  GTTTTAATAATTTTTTTCGGACTAATAATATTTTTTTTGATTAGATTAAATTATCCTTTTTCAAATAGTA
NPR  ............................................................

          290       300       310       320       330       340       350
     ....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TATCATATTTTTATATAAATTTTTTTTTTTTTTTTATAATTTATTCTTTAATAGCTTTTTTCTTTTTACT
NPR  ............................................................

          360       370       380       390       400       410       420
     ....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  ATTAAGTTATAGATCAAATAGATTTTTCTCAATTTTATCAATATTTCGAGTTTTGATTCAAATTATTAGA
NPR  ............................................................

          430       440       450       460       470       480       490
     ....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TATGAAGTTGGATTAATATTTTTATTTTTTTTTCCTTTAATTTTAATAAATATTTTTAATTTTTACTTTT
NPR  ............................................................

          500       510       520       530       540       550       560
     ....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  ACTTCTTTTCTAGAAGTTCAATTATTTTTTTTTCTTTAATTTATATTTATCTTTTAGTTTTAGTTGCGTT
NPR  ............................................................

          570       580       590       600       610       620       630
     ....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  AAGTGAAATAAACCGTTTACCATTTGATTTTCTTGAAAGTGAGACTGAGTTAGTTTCTGGATTTAACATT
NPR  ............................................................
```

```
          640       650       660       670       680       690       700
     ....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  GAGTATATATCTTCTTTATTTAGCTTCATTTTTTTGATTGAGTACGGTTTTTTTTTATATATAATAATAC
NPR  ............................................................................

          710       720       730       740       750       760       770
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TAATTAATTTTTTCTTTTTTTTAAATGATTTTTTTACGTTAATATTGATTTTTTTTGTTATTTGAACTCG
NPR  ............................................................................

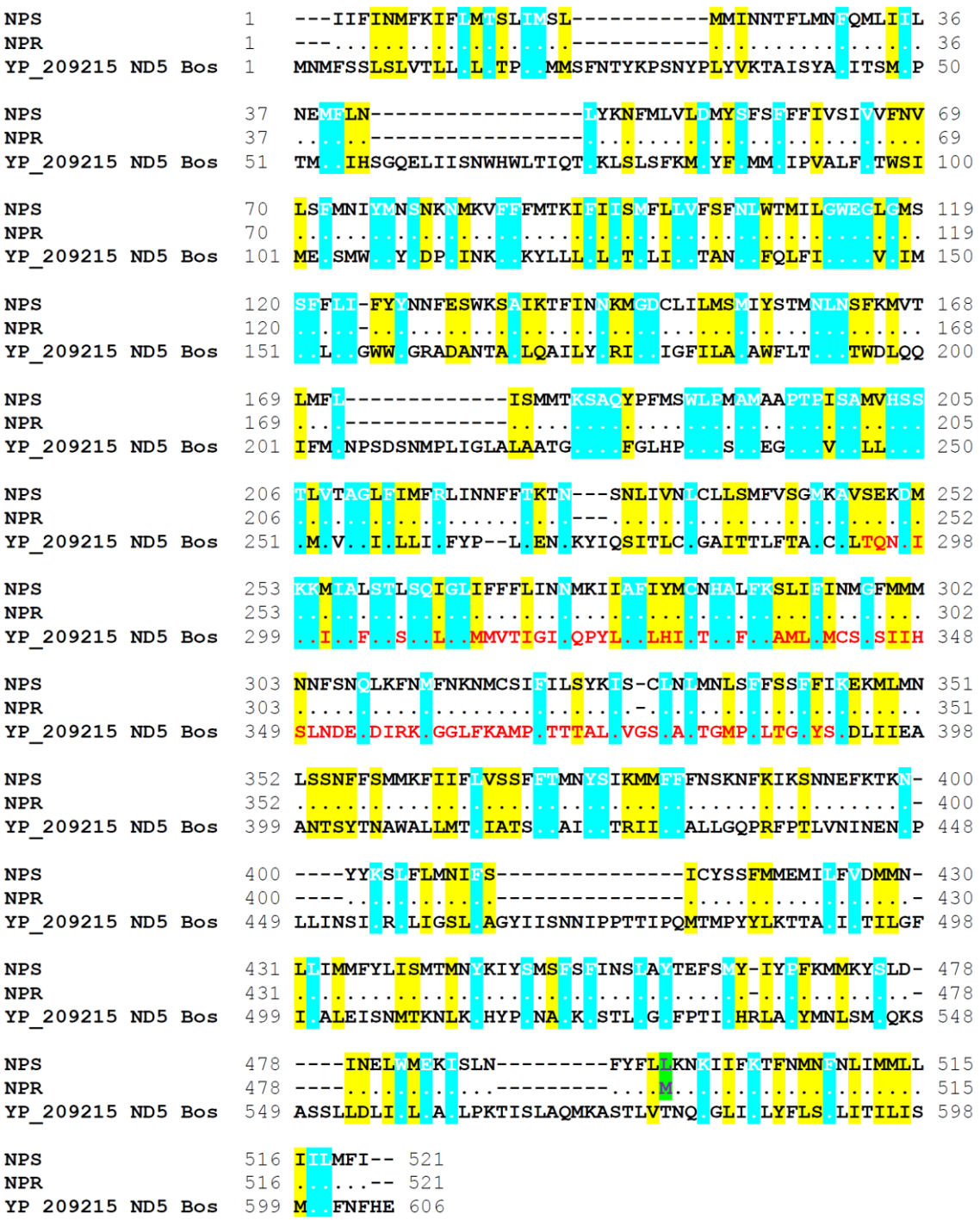
          780       790       800       810       820       830       840
     ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
NPS  TTCATTTTTTCCACGGTTTCGGTATGATAAAATACTATACTTTTTTTGAAAAGAAGTTGTCTTATTAATT
NPR  ............................................................................

          850
     ....|....|....|.
NPS  ATATATCTTTATATTT
NPR  ................
```

Amino acid sequence alignment of NAD5 in NPS and NPR strains with that of Bos taurus (YP_209215 ND5 Bos)

Red font indicates predicted Q-binding domain of ND5 subunit (Nakamura-Ogiso et al. 2003 https://pubs.acs.org/doi/10.1021/bi0269660)

Green shading= L495M mutation in NPR



Supplementary information 4:

Alinement of DNA sequences for CYP392A3 clones of NPR (A3-1–4) and tetur07g06460 (London strain; Grbić et al. 2011)

CLUSTAL 2.1 multiple sequence alignment with some modification

```
A3-1            ----------------------------CCATTACCTTTTATTTACTTGTTCTTGAGTCTG 33
A3-4            ----------------------------CCATTACCTTTTATTTACTTGTTCTTGAGTCTG 33
A3-3            ----------------------------CCATTACCTTTTATTTACTTGTTCTTGAGTCTG 33
A3-2            ----------------------------CCATTACCTTTTATTTACTTGTTCTTGAGTCTG 33
tetur07g06460   ATGTTGCTTCTTGATTACTTTAGTGTGCCATTACCTTTTATTTACTTGTTCTTGAGTCTG 60
                                            ********************************

A3-1            TCCATTATTTGGATCATCAAATATCTTTTCCAATCTGTGAAACGCTTATATTCATTACCA 93
A3-4            TCCATTATTTGGATCATCAAATATCTTTTCCAATCTGTGAAACGCTTATATTCATTACCA 93
A3-3            TCCATTATTTGGATCATCAAATATCTTTTCCAATCTGTGAAACGCTTATATTCATTACCA 93
A3-2            TCCATTATTTGGATCATCAAATATCTTTTCCAATCTGTGAAACGCTTATATTCATTACCA 93
tetur07g06460   TCCATTATTTGGATCATCAAATATCTTTTCAAATCTGTGAAACGAATATATTCATTACCT 120
                ****************************** ************  ************

A3-1            CCAGGACCATTTGGAATTCCAATATTTGGTTATTACCCGTTCTTGAAACATCACAGTTAC 153
A3-4            CCAGGACCATTTGGAATTCCAATATTTGGTTATTACCCGTTCTTGAAACATCACAGTTAC 153
A3-3            CCAGGACCATTTGGAATTCCAATATTTGGTTATTACCCGTTCTTGAAACATCACAGTTAC 153
A3-2            CCAGGACCATTTGGAATTCCAATATTTGGTTATTACCCGTTCTTGAAACATCACAGTTAC 153
tetur07g06460   CCAGGACCATTTGGATTTCCGATTTTTGGTTATTATCCGTTCTTAAAGGATCATAGTTAT 180
                *************** **** ** ********** ******** **  **** *****

A3-1            ATACAATTCGATCGACTATCTAAAAAGTATGGACCAGTTTTCAGTCTAAAGTTGGGTCAA 213
A3-4            ATACAATTCGATCGACTATCTAAAAAGTATGGACCAGTTTTCAGTCTAAAGTTGGGTCAA 213
A3-3            ATACAATTCGATCGACTATCTAAAAAGTATGGACCAGTTTTCAGTCTAAAGTTGGGTCAA 213
A3-2            ATACAATTCGATCGACTATCTAAAAAGTATGGACCAGTTTTCAGTCTAAAGTTGGGTCAA 213
tetur07g06460   ATACAATTTGATCGACTATCCAAAAAGTATGGACCAGTTTTCAGTCTAAAGTTGGGTCAA 240
                ******** ********** ************************************

A3-1            TATGATACTATTGTTGTCTGTGATTGGGATAACCTTAAAGACGCCTTCGCAAATGATGCT 273
A3-4            TATGATACTATTGTTGTCTGTGATTGGGATAACCTTAAAGACGCCTTCGCAAATGATGCT 273
A3-3            TATGATACTATTGTTGTCTGTGATTGGGATAACCTTAAAGACGCCTTCGCAAATGATGCT 273
A3-2            TATGATACTATTGTTGTCTGTGATTGGGATAACCTTAAAGACGCCTTCGCAAATGATGCT 273
tetur07g06460   TATGATACTATTGTTGTCTGTGATTGGGATAACCTTAAAGATGCCTTCGCAAATGATGCT 300
                ************************************** ******************

A3-1            TTATTGGCTCGTCATGCTAAAGGTTTCTTATCAGGAATAGAGAACACGCTTTCGATTATT 333
A3-4            TTATTGGCTCGTCCTGCTAAAGGTTTCTTATCAGGAATAGAGAACACGCTTTCGATTATT 333
A3-3            TTATTGGCTCGTCCTGCTAAAGGTTTCTTATCAGGAATAGAGAACACGCTTTCGATTATT 333
A3-2            TTATTGGCTCGTCCTGCTAAAGGTTTCTTATCAGGAATAGAGAACACGCTTTCGATTATT 333
tetur07g06460   TTATTGGCTCGTCCTGCTAAAGGTTTCTTATCAGGAATAGAGAACACGCTTTCGATTATT 360
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A3-1              TCAATGTCGGGTGATGCTTGGCGTGAACACAGACGGTTGTCATTACATGTTTTGCGTAAT 393
A3-4              TCAATGTCGGGTGATGCTTGGCGTGAACACAGACGGTTGTCATTACATGTTTTGCGTAAT 393
A3-3              TCAATGTCGGGTGATGCTTGGCGTGAACACAGACGGTTGTCATTACATGTTTTGCGTAAT 393
A3-2              TCAATGTCGGGTGATGCTTGGCGTGAACACAGACGGTTGTCATTACATGTTTTGCGTAAT 393
tetur07g06460     TCAATGTCGGGTGATGCTTGGCGTGAACATAGACGGTTGTCATTACATGTTTTGCGTAAT 420
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A3-1              GTTGGTTTGGGTAAACGAGAAATGGAAACTTTGATCTCGGAAGAAATTCATCAATTTTTG 453
A3-4              GTTGGTTTGGGTAAACGAGAAATGGAAACTTTGATCTCGGAAGAAATTCATCAATTTTTG 453
A3-3              GTTGGTTTGGGTAAACGAGAAATGGAAACTTTGATCTCGGAAGAAATTCATCAATTTTTG 453
A3-2              GTTGGTTTGGGTAAACGAGAAATGGAAACTTTGATCTCGGAAGAAATTCATCAATTTTTG 453
tetur07g06460     GTTGGTTTGGGTAAACGAGAAATGGAAAATTTGATCTCGGAAGAAATTCACCAATTTTTG 480
                  *************************** ******************** *********

A3-1              TCTTCACTTGAAAATGATGCAAATGACTTGCCTCAACGTCTAATGCAAAGTGTCTCTAAT 513
A3-4              TCTTCACTTGAAAATGATGCAAATGACTTGCCTCAACGTCTAATGCAAAGTGTCTCTAAT 513
A3-3              TCTTCACTTGAAAATGATGCAAATGACTTGCCTCAACGTCTAATGCAAAGTGTCTCTAAT 513
A3-2              TCTTCACTTGAAAATGATGCAAATGACTTGCCTCAACGTCTAATGCAAAGTGTCTCTAAT 513
tetur07g06460     TATTCACTTGAAAATGATGCGAATGACTTGTCTCAACGTCTAATGCAAAGTGTCTCTAAT 540
                  * ***************** ********* ****************************

A3-1              AACATTTCGCTTATGCTATTTGGTCATATTTTTGATTATGATGATCCAGATAAAGTGGCG 573
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A3-3              AACATTTCGCTTATGCTATTTGGTCATATTTTTGATTATGATGATCCAGATAAAGTGGCG 573
A3-2              AACATTTCGCTTATGCTATTTGGTCATATTTTTGATTATGATGATCCAGATAAAGTGGCG 573
tetur07g06460     AACATTTCGCTTATGCTATTTGGTCATATTTTTGATTATGATGATCCAGATAAAGTGGCG 600
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A3-1              ATTGATGAAAGTCTGAGAGATTATTGCCAAGCATTTCAATTTTCAGGCATAACGAGCTAT 633
A3-4              ATTGATGAAAGTCTGAGAGATTATTGCCAAGCATTTCAATTTTCAGGCATAACGAGCTAT 633
A3-3              ATTGATGAAAGTCTGAGAGATTATTGCCAAGCATTTCAATTTTCAGGCATAACGAGCTAT 633
A3-2              ATTGATGAAAGTCTGAGAGATTATTGCCAAGCATTTCAATTTTCAGGCATAACGAGCTAT 633
tetur07g06460     ATTGATGAAAGTCTGAGAGATTATTGCCAAGCATTTCAATTTTCAGGCATAACGAGCTAT 660
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A3-1              TTACCATGGTTAACTAAACCCTTAATTGCTTTAGGTAAAGCTAACCTTAAAATTATTCAG 693
A3-4              TTACCATGGTTAACTAAACCCTTAATTGCTTTAGGTAAAGCTAACCTTAAAATTATTCAG 693
A3-3              TTACCATGGTTAACTAAACCCTTAATTGCTTTAGGTAAAGCTAACCTTAAAATTATTCAG 693
A3-2              TTACCATGGTTAACTAAACCCTTAATTGCTTTAGGTAAAGCTAACCTTAAAATTATTCAG 693
tetur07g06460     TTACCATGGTTAACTAAACCACTGATTGCTTTAGGTAAAGCTAACCTTAAAATTATACAA 720
                  ******************  * ******************************** **

A3-1              AAAGCTCAAATGCATCTAAACGACTTCATTTCAAAAGAGCTTTTCAAACACCAGAATGCG 753
A3-4              AAAGCTCAAATGCATCTAAACGACTTCATTTCAAAAGAGCTTTTCAAACACCAGAATGCG 753
A3-3              AAAGCTCAAATGCATCTAAACGACTTCATTTCAAAAGAGCTTTTCAAACACCAGAATGCG 753
A3-2              AAAGCTCAAATGCATCTAAACGACTTCATTTCAAAAGAGCTTTTCAAACACCAGAATGCG 753
tetur07g06460     AAAGCTCAAATGCATCTAAACGACTTCATTTCAAAAGAGCTTTTCAAACACCAGAATGGG 780
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                        INS1
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A3-3              ACAGATGCGTTACAAATTGAAAACTACATTGATGGATACTTAAATGTCCAGTCTAAGCGG 813
A3-2              ACAGATGCGTTACAAATTGAAAACTACATTGATGGATACTTAAATGTCCAGTCTAAGCGG 813
tetur07g06460     AAA----------GTTGAAAACTACATTGATGGATACTTAAATGTCCAGTCTAAGCGG 828
                  * *            ****************************************
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A3-1              AAAGATCAACTATTCAATAATGCAACATTAAAAAGGAATGTTGCCACATTCTTTGTTGCA 873
A3-4              AAAGATCAACTATTCAATAATGCAACATTAAAAAGGAATGTTGCCACATTCTTTGTTGCA 873
A3-3              AAAGATCAACTATTCAATAATGCAACATTAAAAAGGAATGTTGCCACATTCTTTGTTGCA 873
A3-2              AAAGATCAACTATTCAATAATGCAACATTAAAAAGGAATGTTGCCACATTCTTTGTTGCA 873
tetur07g06460     AAAGATCAACTATTCAATAATGCAACATTAAAAAGGAATGTTGCCACATTCTTTGTTGCA 888
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A3-1              GGATCAGAGACCGTTGCCGGTACTCTAACCTGGGCCAGCATGTACCTAGTAAAATATCCT 933
A3-4              GGATCAGAGACCGTTGCCGGTACTCTAACCTGGGCCAGCATGTACCTAGTAAAATATCCT 933
A3-3              GGATCAGAGACCGTTGCCGGTACTCTAACCTGGGCCAGCATGTACCTAGTAAAATATCCT 933
A3-2              GGATCAGAGACCGTTGCCGGTACTCTAACCTGGGCCAGCATGTACCTAGTAAAATATCCT 933
tetur07g06460     GGATCAGAGACCGTTGCCGGTACTCTAACCTGGGCCAGCATGTACCTAGTAAAATATCCT 948
                  ************************************************************

A3-1              CAATATCAAGAGAGGATTCGATTAGAAATAAAAGAAGTTATTGGAACTGAAAAGAGACCA 993
A3-4              CAATATCAAGAGAGGATTCGATTAGAAATAAAAGAAGTTATTGGAACTGAAAAGAGACCA 993
A3-3              CAATATCAAGAGAGGATTCGATTAGAAATAAAAGAAGTTATTGGAACTGAAAAGAGACCA 993
A3-2              CAATATCAAGAGAGGATTCGATTAGAAATAAAAGAAGTTATTGGAACTGAAAAGAGACCA 993
tetur07g06460     CAATATCAAGAGAGGATTCGATTAGAAATAAAAGAAGTTATTGGAACTGAAAAACGACCA 1008
                  ************************************************  *****

A3-1              GATTTCTCGGATCGTCTAAGAATGCCTTTTACTCTAGCCTTTCTTTATGAAGTCCAGCGT 1053
A3-4              GATTTCTCGGATCGTCTAAGAATGCCTTTTACTCTAGCCTTTCTTTATGAAGTCCAGCGT 1053
A3-3              GATTTCTCGGATCGTCTAAGAATGCCTTTTACTCTAGCCTTTCTTTATGAAGTCCAGCGT 1053
A3-2              GATTTCTCGGATCGTCTAAGAATGCCTTTTACTCTAGCCTTTCTTTATGAAGTCCAGCGT 1053
tetur07g06460     GATTTCTCGGATCGTCTAAGAATGCCTTTTACTCTCGCCTTTCTTTATGAAGTCCAGCGT 1068
                  ********************************** ************************

A3-1              ATTGAGTCAATCGTGGCTACAAATCTTATACGAAGAGCTTCACAAGACACAAAGATTGGT 1113
A3-4              ATTGAGTCAATCGTGGCTACAAATCTTATACGAAGAGCTTCACAAGACACAAAGATTGGT 1113
A3-3              ATTGAGTCAATCGTGGCTACAAATCTTATACGAAGAGCTTCACAAGACACAAAGATTGGT 1113
A3-2              ATTGAGTCAATCGTGGCTACAAATCTTATACGAAGAGCTTCACAAGACACAAAGATTGGT 1113
tetur07g06460     ATTGAATCCATCGTCGCTACAAATCTTATACGAAGAGCTTCACAAGACACAAAGATTGGT 1128
                  ***** ** ***** ********************************************

A3-1              CCTTATAATGTTCCAAAAGATAGTCTGGTTCTGTTCAATTTCTGGTCCGTCCATCATGAT 1173
A3-4              CCTTATAATGTTCCAAAAGATAGTCTGGTTCTGTTCAATTTCTGGTCCGTCCATCATGAT 1173
A3-3              CCTTATAATGTTCCAAAAGATAGTCTGGTTCTGTTCAATTTCTGGTCCGTCCATCATGAT 1173
A3-2              CCTTATAATGTTCCAAAAGATAGTCTGGTTCTGTTCAATTTCTGGTCCGTCCATCATGAT 1173
tetur07g06460     CCTTATAATGTTCCAAAAGATAGTCTGGTTCTGTTCAATTTCTGGTCTGTCCATCATGAT 1188
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                                                                    INS2
A3-1              CCCAAACTGTGGCCTAATCCTGATAAATTTGATCCAAATCGATTCCTTGCTGAAAATATT 1233
A3-4              CCCAAACTGTGGCCTAATCCTGATAAATTTGATCCAAATCGATTCCTTGCTGAAAATATT 1233
A3-3              CCCAAACTGTGGCCTAATCCTGATAAATTTGATCCAAATCGATTCCTTGCTGAAAATATT 1233
A3-2              CCCAAACTGTGGCCTAATCCTGATAAATTTGATCCAAATCGATTCCTTGCTGAAAATATT 1233
tetur07g06460     CCAAAACTTTGGTCTAATCCCGATAAATTTGATCCAAATCGATTCCTTACCGAA------ 1242
                  ** ***** *** ******* *************************** * ***

A3-1              ACCAATAGTACCAAAGTAATAAAGTCTCCATATTTAGTGCCATTTAGTGCTGGTAAAAGA 1293
A3-4              ACCAATAGTACCAAAGTAATAAAGTCTCCATATTTAGTGCCATTTAGTGCTGGTAAAAGA 1293
A3-3              ACCAATAGTACCAAAGTAATAAAGTCTCCATATTTAGTGCCATTTAGTGCTGGTAAAAGA 1293
A3-2              ACCAATAGTACCAAAGTAATAAAGTCTCCATATTTAGTGCCATTTAGTGCTGGTAAAAGA 1293
tetur07g06460     ---AATGGTAACAAAGTAGTAAAGCCTCCATATTTAGTGCCATTTAGTGCTGGTAAAAGA 1299
                     *** *** ******* ***** ********************************
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A3-1              GCTTGTCCAGGTGAAGGCTTAGCTAATGTGGAGCTATTTTTGTACACAGTTGGTATACTT 1353
A3-4              GCTTGTCCAGGTGAAGGCTTAGCTAATGTGGAGCTATTTTTGTACACAGTTGGTATACTT 1353
A3-3              GCTTGTCCAGGTGAAGGCTTAGCTAATGTGGAGCTATTTTTGTACACAGTTGGTATACTT 1353
A3-2              GCTTGTCCAGGTGAAGGCTTAGCTAATGTGGAGCTATTTTTGTACACAGTTGGTATACTT 1353
tetur07g06460     GCTTGTCCAGGTGAAGGCTTAGCTAATGTGGAGCTATTTTTATACACAGTTGGTATACTT 1359
                  ************************************** ******************

A3-1              CAACGATTCAAAATCAAATCAGACAAACCATTGTCATTTGAAGCAATTAACGGTCTCACT 1413
A3-4              CAACGATTCAAAATCAAATCAGACAAACCATTGTCATTTGAAGCAATTAACGGTCTCACT 1413
A3-3              CAACGATTCAAAATCAAATCAGACAAACCATTGTCATTTGAAGCAATTAACGGTCTCACT 1413
A3-2              CAACGATTCAAAATCAAATCAGACAAACCATTGTCATTTGAAGCAATTAACGGTCTCACT 1413
tetur07g06460     CAACGATTCAAAATCAAATCAGATAAACCATTATCCTTTGAAGCAATTAACGGTCTCACT 1419
                  ********************** ******** ** ************************

A3-1              CGACGTCCTAAATACAAA---------------------------- 1431
A3-4              CGACGTCCTAAATACAAA---------------------------- 1431
A3-3              CGACGTCCTAAATACAAA---------------------------- 1431
A3-2              CGACGTCCTAAATACAAA---------------------------- 1431
tetur07g06460     CGACGTCCAAAATACAAACCAGATTTAATCTTCCAAAGAGTATAA 1464
                  ******** *****
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Supplementary information 5:

Alinement of amino acid sequences for CYP392A3 clones of NPR (A3-1–4) and tetur07g06460 (London strain; Grbić et al. 2011)

CLUSTAL 2.1 multiple sequence alignment with some modification

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A3-1              ---------PLPFIYLFLSLSIIWIIKYLFQSVKRLYSLPPGPFGIPMFGYYPFLKHHSY 51
A3-4              ---------PLPFIYLFLSLSIIWIIKYLFQSVKRLYSLPPGPFGIPMFGYYPFLKHHSY 51
A3-3              ---------PLPFIYLFLSLSIIWIIKYLFQSVKRLYSLPPGPFGIPMFGYYPFLKHHSY 51
A3-2              ---------PLPFIYLFLSLSIIWIIKYLFQSVKRLYSLPPGPFGIPMFGYYPFLKHHSY 51
tetur07g06460     MLLLDYFSVPLPFIYLFLSLSIIWIIKYLFKSVKRIYSLPPGPFGFPIFGYYPFLKDHSY 60
                          ********************* **** ********* * ******** ***

A3-1              MQFDRLSKKYGPVFSLKLGQYDTIVVCDWDNLKDAFANDALLARHAKGFLSGMENTLSII 111
A3-4              MQFDRLSKKYGPVFSLKLGQYDTIVVCDWDNLKDAFANDALLARPAKGFLSGMENTLSII 111
A3-3              MQFDRLSKKYGPVFSLKLGQYDTIVVCDWDNLKDAFANDALLARPAKGFLSGMENTLSII 111
A3-2              MQFDRLSKKYGPVFSLKLGQYDTIVVCDWDNLKDAFANDALLARPAKGFLSGMENTLSII 111
tetur07g06460     IQFDRLSKKYGPVFSLKLGQYDTIVVCDWDNLKDAFANDALLARPAKGFLSGIENTLSII 120
                   ****************************************** ******* *******

A3-1              SMSGDAWREHSRLSLHVLRNVGLGKREMETLISEEIHQFLSSLENDANDLPQRLMQSVSN 171
A3-4              SMSGDAWREHSRLSLHVLRNVGLGKREMETLISEEIHQFLSSLENDANDLPQRLMQSVSN 171
A3-3              SMSGDAWREHSRLSLHVLRNVGLGKREMETLISEEIHQFLSSLENDANDLPQRLMQSVSN 171
A3-2              SMSGDAWREHSRLSLHVLRNVGLGKREMETLISEEIHQFLSSLENDANDLPQRLMQSVSN 171
tetur07g06460     SMSGDAWREHRRLSLHVLRNVGLGKREMENLISEEIHQFLYSLENDANDLSQRLMQSVSN 180
                  ********** ***************** ********** ********* *********

A3-1              NISLMLFGHIFDYDDPDKVAIDESLSDYCQAFQFSGMTSYLPWLTKPLIALGKANLKIIQ 231
A3-4              NISLMLFGHIFDYDDPDKVAIDESLSDYCQAFQFSGMTSYLPWLTKPLIALGKANLKIIQ 231
A3-3              NISLMLFGHIFDYDDPDKVAIDESLSDYCQAFQFSGMTSYLPWLTKPLIALGKANLKIIQ 231
A3-2              NISLMLFGHIFDYDDPDKVAIDESLSDYCQAFQFSGMTSYLPWLTKPLIALGKANLKIIQ 231
tetur07g06460     NISLMLFGHIFDYDDPDKVAIDESLRDYCQAFQFSGITSYLPWLTKPLIALGKANLKIIQ 240
                  ************************ ********** ************************

                                    p-INS1
A3-1              KAQMHLNDFISKELFKHQNATDALQIENYIDGYLNVQSKRKDQLFNNATLKSNVATFFVA 291
A3-4              KAQMHLNDFISKELFKHQNATDALQIENYIDGYLNVQSKRKDQLFNNATLKSNVATFFVA 291
A3-3              KAQMHLNDFISKELFKHQNATDALQIENYIDGYLNVQSKRKDQLFNNATLKSNVATFFVA 291
A3-2              KAQMHLNDFISKELFKHQNATDALQIENYIDGYLNVQSKRKDQLFNNATLKSNVATFFVA 291
tetur07g06460     KAQMHLNDFISKELFKHQNGK----VENYIDGYLNVQSKRKDQLFNNATLKRNVATFFVA 296
                  ******************       ************************ ********

A3-1              GSETVAGTLTWASMYLVKYPQYQESIRLEMKEVIGTEKSPDFSDRLSMPFTLAFLYEVQR 351
A3-4              GSETVAGTLTWASMYLVKYPQYQESIRLEMKEVIGTEKSPDFSDRLSMPFTLAFLYEVQR 351
A3-3              GSETVAGTLTWASMYLVKYPQYQESIRLEMKEVIGTEKSPDFSDRLSMPFTLAFLYEVQR 351
A3-2              GSETVAGTLTWASMYLVKYPQYQESIRLEMKEVIGTEKSPDFSDRLSMPFTLAFLYEVQR 351
tetur07g06460     GSETVAGTLTWASMYLVKYPQYQERIRLEIKEVIGTEKRPDFSDRLRMPFTLAFLYEVQR 356
                  ********************** **** ******** ******* *************
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                                                                  p-INS2
A3-1              IESIVATNLMRSASQDTKIGPYNVPKDSLVLFNFWSVHHDPKLWPNPDKFDPNRFLAENI 411
A3-4              IESIVATNLMRSASQDTKIGPYNVPKDSLVLFNFWSVHHDPKLWPNPDKFDPNRFLAENI 411
A3-3              IESIVATNLMRSASQDTKIGPYNVPKDSLVLFNFWSVHHDPKLWPNPDKFDPNRFLAENI 411
A3-2              IESIVATNLMRSASQDTKIGPYNVPKDSLVLFNFWSVHHDPKLWPNPDKFDPNRFLAENI 411
tetur07g06460     IESIVATNLIRRASQDTKIGPYNVPKDSLVLFNFWSVHHDPKLWSNPDKFDPNRFLTE-- 414
                  ********* * ******************************** ********** *

A3-1              TNSTKVMKSPYLVPFSAGKSACPGEGLANVELFLYTVGMLQRFKIKSDKPLSFEAINGLT 471
A3-4              TNSTKVMKSPYLVPFSAGKSACPGEGLANVELFLYTVGMLQRFKIKSDKPLSFEAINGLT 471
A3-3              TNSTKVMKSPYLVPFSAGKSACPGEGLANVELFLYTVGMLQRFKIKSDKPLSFEAINGLT 471
A3-2              TNSTKVMKSPYLVPFSAGKSACPGEGLANVELFLYTVGMLQRFKIKSDKPLSFEAINGLT 471
tetur07g06460     -NGNKVVKPPYLVPFSAGKRACPGEGLANVELFLYTVGILQRFKIKSDKPLSFEAINGLT 473
                   *  ** * ********** ****************** **********************

A3-1              RRPKYK-------- 477
A3-4              RRPKYK-------- 477
A3-3              RRPKYK-------- 477
A3-2              RRPKYK-------- 477
tetur07g06460     RRPKYKPDLIFQRV 487
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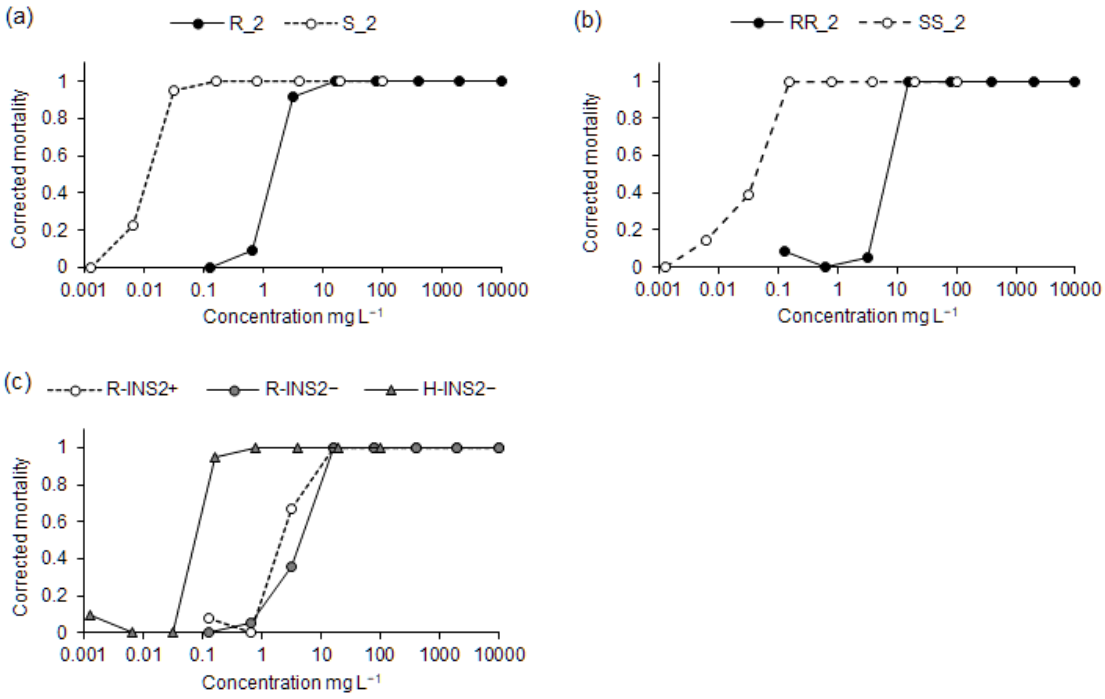
Supplementary information 6:

Twenty-five adult females were introduced to a kidney bean leaf disk (3 × 3 cm). The leaf disk was sprayed with 0.3 mL PBO solution (100 mg L^{-1} in a solvent of acetone:water = 1:1) using a glass chromatograph sprayer. Four hours later, the leaf disk was sprayed with pyridaben solution 1 mL at assigned concentration. Mortality was checked after 24 h. Pyridaben application and assessment were performed as well as toxicological bioassay (section 2.6.1). Consequently, 100% of R_2, RR_2, R-INS2+, and R-INS2− females died at the concentration of > 16 mg L^{-1}. All females died at > 0.16 mg L^{-1} in S_2 and SS_2 and > 0.8 mg L^{-1} in H-INS2−. Therefore, marked synergistic effect on pyridaben toxicity was shown in all congenic strains by the inhibition of CYP activity with PBO pretreatment.



Mortality–dose curve for adult females sprayed with pyridaben after PBO pretreatment. (a) R_2 and S_2 strains, (b) RR_2 and SS_2 strains, (c) R-INS2+, R-INS2−, and H-INS2− strains.

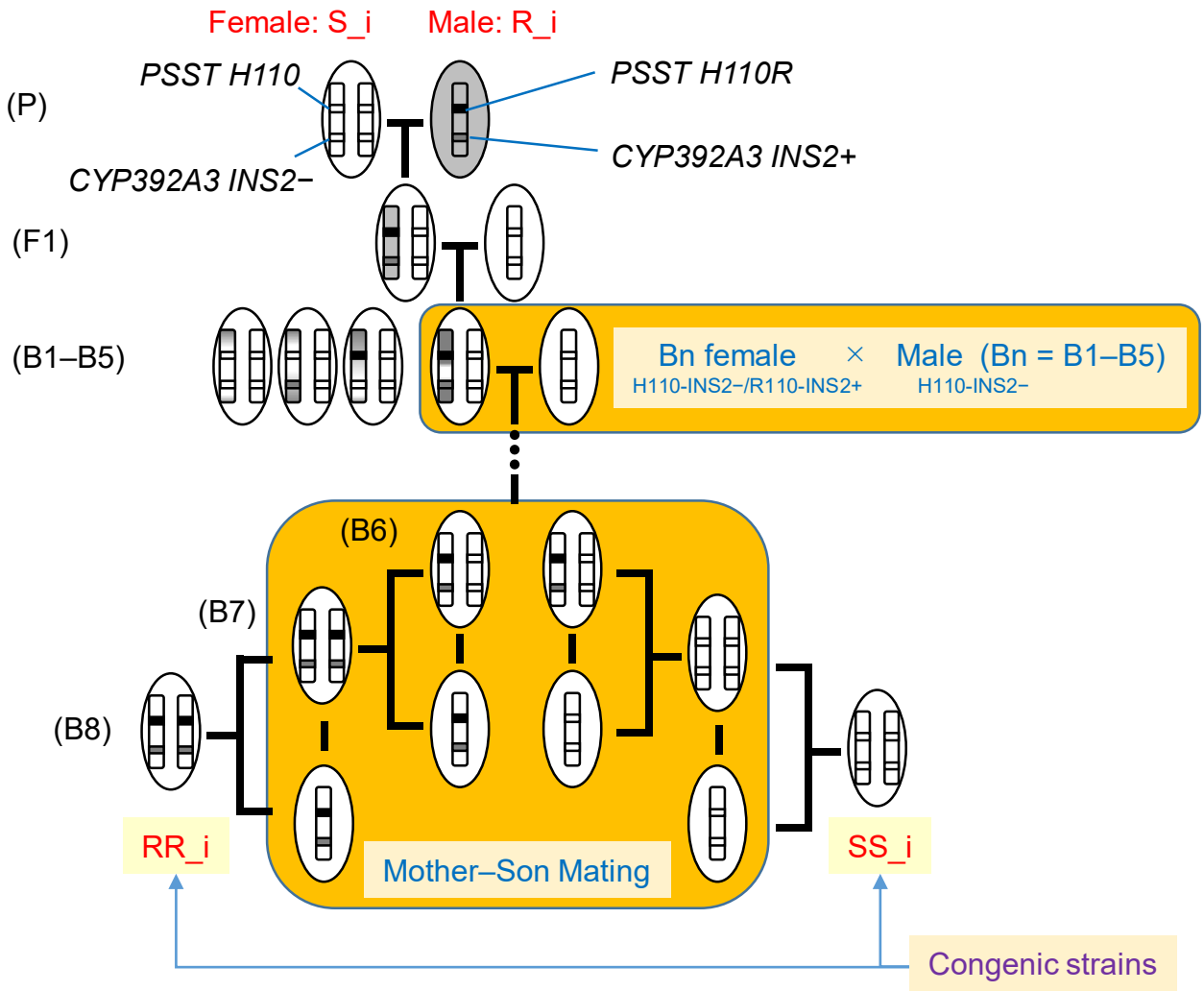


Fig. S1. Procedure of the marker-assisted back crossing for double introgression of mutant PSST and high-expression CYP392A3 alleles into susceptible strain. R_i (i = 1, 2): single-female lines of NPR that had alleles of c.329A>G (H110R) in *PSST* and c.1242_1243insAATATTACC (p.E414_N415insNIT; INS2+ type; see section 3.7) in *CYP392A3* homogeneously. S_i (i = 1, 2): single-female lines of NPS that had alleles of 329A (H110) in PSST and no insertion (c.1246_1247; INS2− type) in CYP392A3 homogeneously

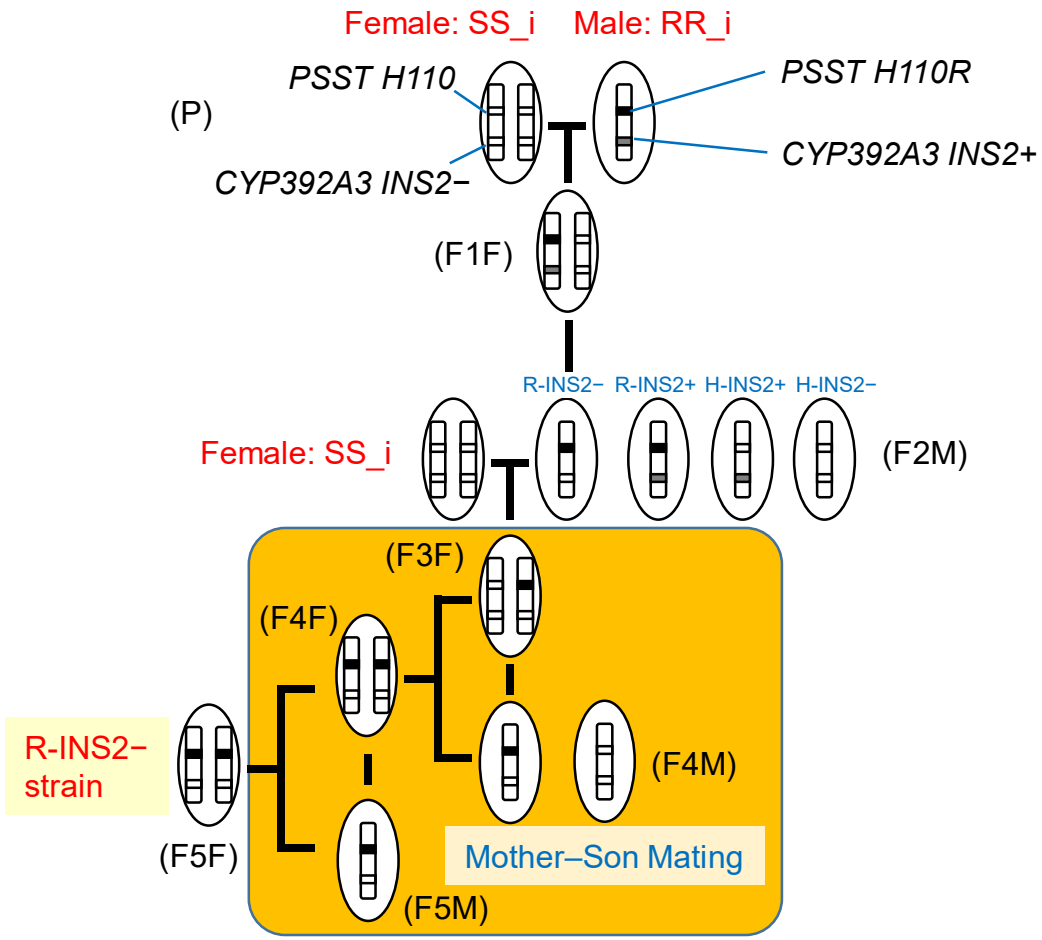


Fig. S2. Procedure of the marker-assisted crossing for uncoupling of mutant *PSST* and INS2+ type *CYP392A3* (see section 3.7) alleles in RR_i (i = 1, 2) constructed in section 2.8.1. This Figure shows the procedure to establish R-INS2− (*PSST* H110R + *CYP392A3* INS2− type) strain as an example.

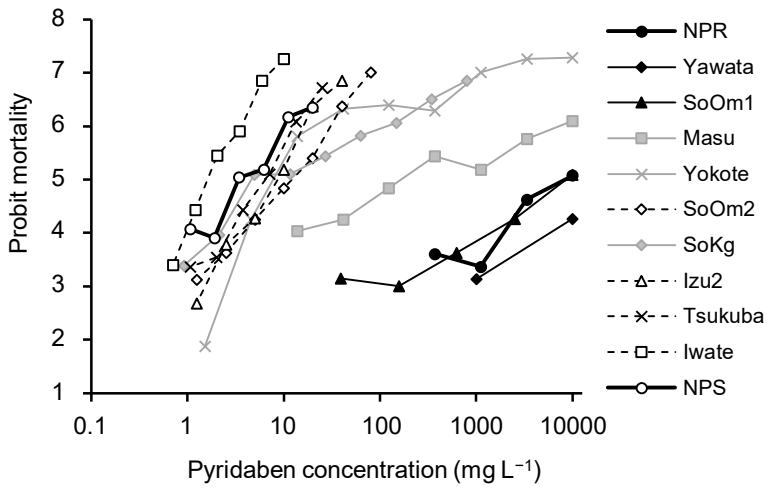


Fig. S3. Pyridaben concentration–mortality curves for *T. urticae* populations.

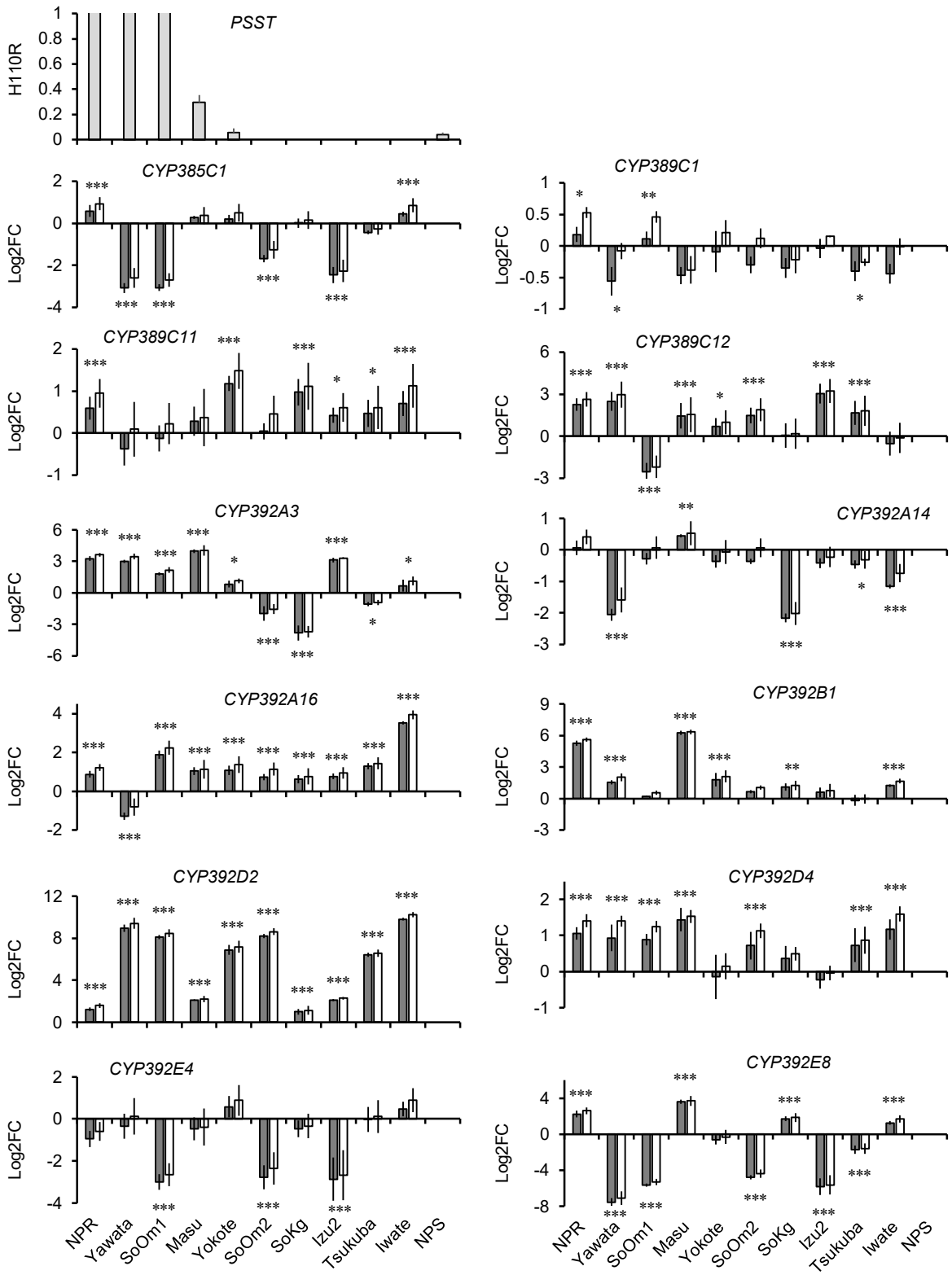


Fig. S4. Frequency of H110R (= $2^{-\Delta\Delta Cq}$) in *PSST* and log2-fold change (Log2FC = $-\Delta\Delta Cq$) of expression levels against NPS in *CYP* genes up-regulated in RNA-seq in the 11 *T. urticae* strains. In CYPs, gray and white bars shows log2FC calculated using GAPDH and ubiquitin as reference genes, respectively. Asterisks above and below bars indicate significant difference from NPS in the ΔCq values (Dunnett contrasts; *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$)

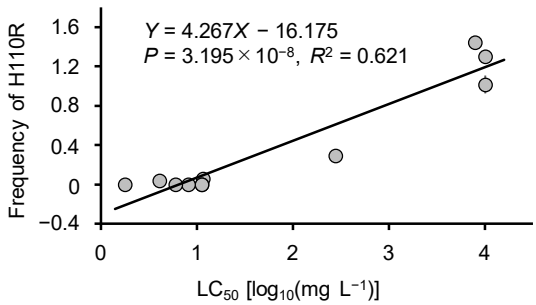


Fig. S5 Regression analysis between the LC_{50} for pyridaben and H110R substitution frequency in PSST genes in the 11 *T. urticae* strains. Bars on the plots show SE.

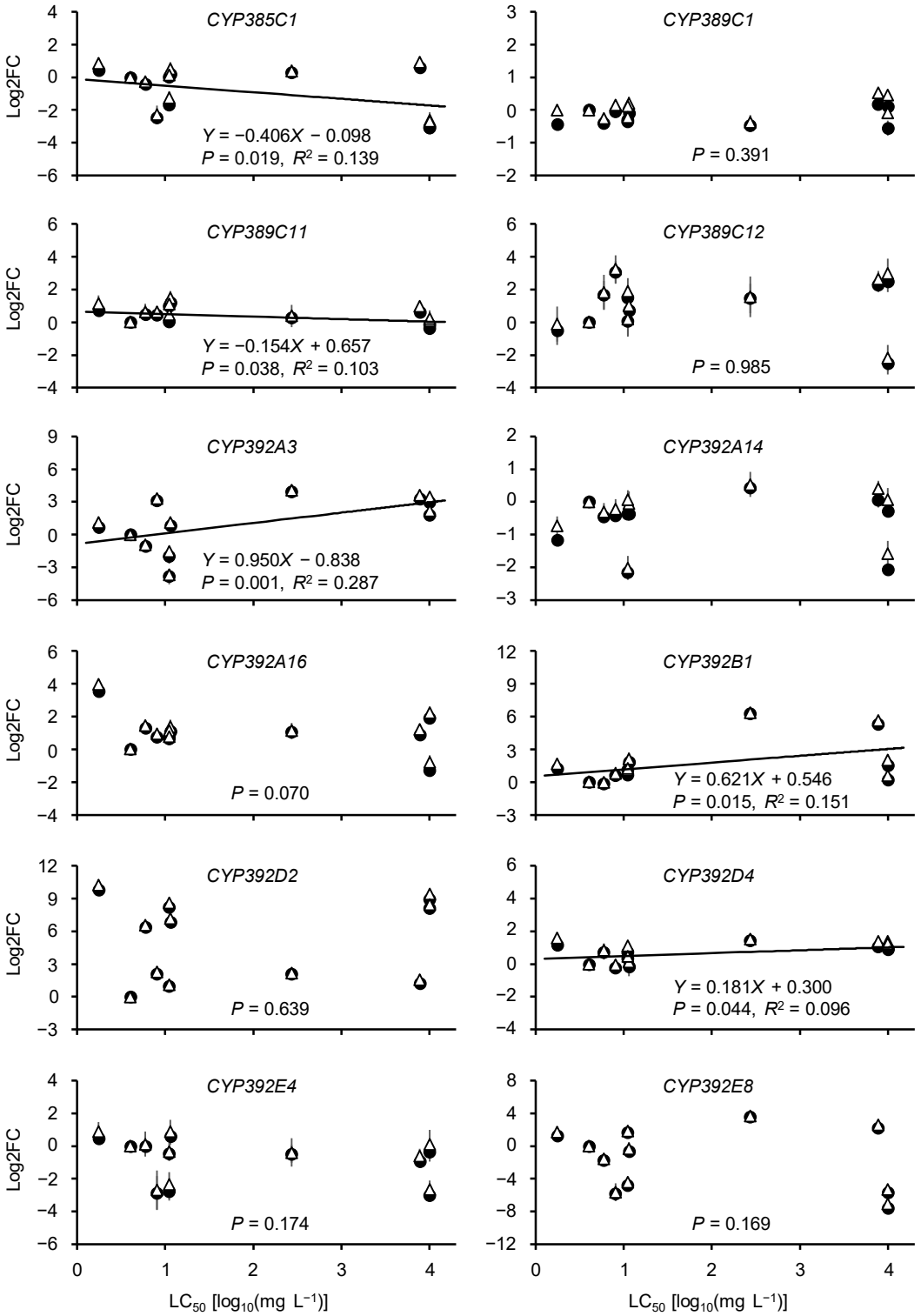


Fig. S6. Regression analysis between the LC_{50} for pyridaben and log2-fold change (Log2FC = $-\Delta\Delta Cq$)) in the expression level of 12 CYP genes up-regulated in RNA-seq in the 11 *T. urticae* strains. Solid circles and open triangles indicate the expression levels computed using GAPDH and ubiquitin as references. Vertical bars on the plots show SE.

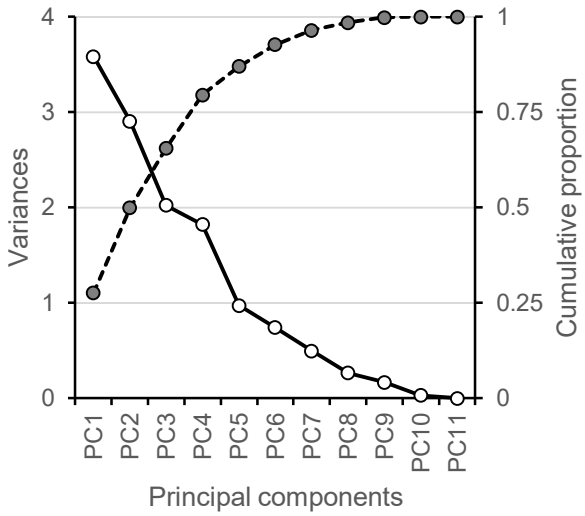


Fig. S7. Variances (open circles) and cumulative contribution (solid circles) of principal components.

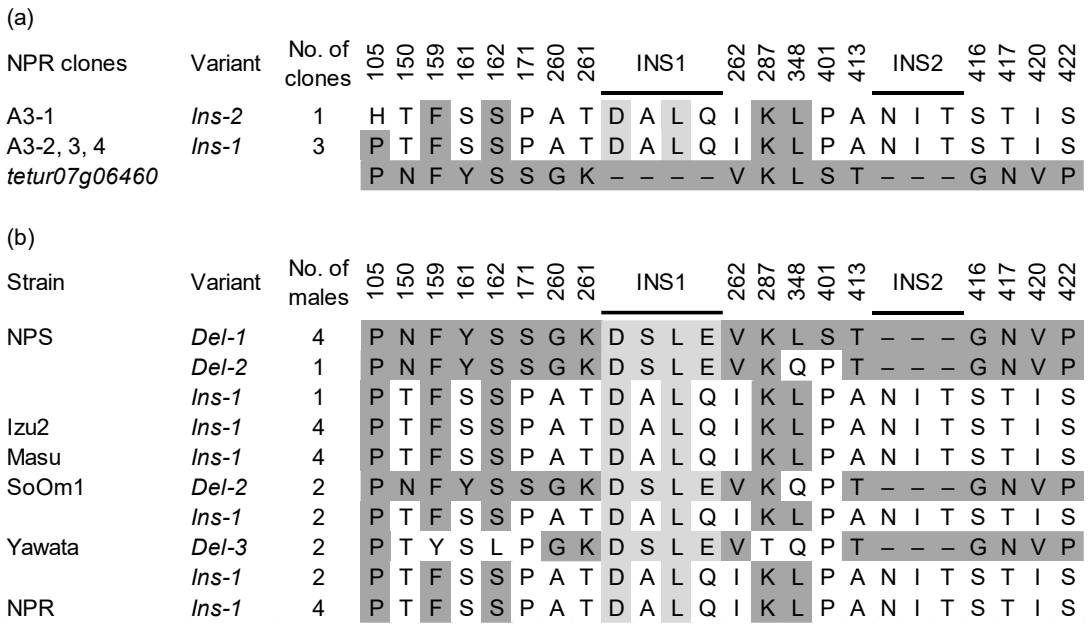


Fig. S8. Insertion (INS1 and INS2) and substitutions of amino acid residues in *CYP392A3*. (a) Comparison between the standard London strain (*tetur07g06460*) and *CYP392A3* of NPR cloned into plasmids (A3-1–4). (b) Amino acid sequences determined from males of local populations. Numbers shown above amino acid residues indicate the numbering for *tetur07g06460*. INS1: p.K261T_V262Iins, INS2: E414_N415ins.

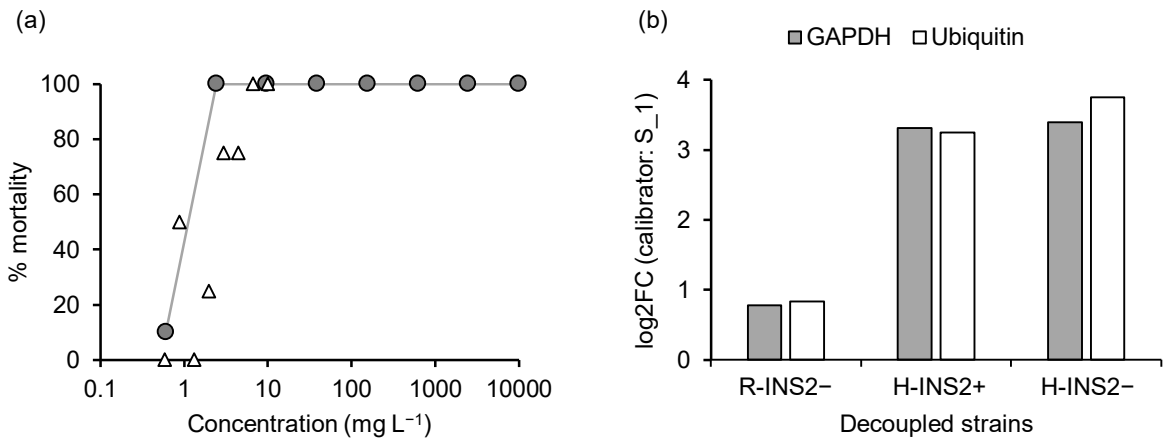


Fig. S9. Mortalities by pyridaben (a) and CYP392A3 expression (b) in H-INS2+ line. (a) Numbers of females used for each concentration were 10.4 ± 0.5 (gray circles) and 3.8 ± 0.4 (open triangle). (b) Hold change was computed using S_1 (ΔCq) as a calibrator with no replication. The experiment of qPCR was performed once separately from that in Fig. 7. Although a sample of R-INS2+ was also applied for qPCR, data was not available due to technical error.

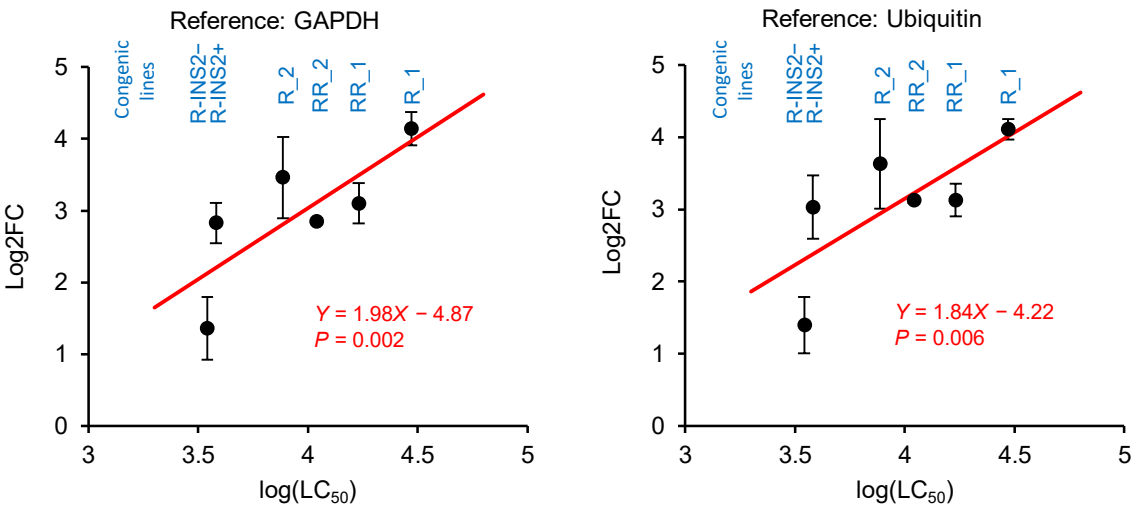


Fig. S10. Correlation between LC_{50} values to pyridaben and gene expression levels (Log2FC) of *CYP392A3* in congenic lines having H110R mutation in *PSST*. Log2FC of all lines were re-computed using S_1 as a common calibrator and *GAPDH* and *ubiquitin* as reference genes. Plots express averaged Log2FC for three biological replications per congenic line and vertical bars show standard errors. Each biological replication (three per congenic line) was used as a sample for the linear regression analysis, which was performed using 'lm' module of R software.

Assuming only the effects of mutant PSST without *CYP392A3* and thus given the expression level decreased to that equivalent to S_1, estimated LC_{50} values based on the regression lines were 290 and 195 mg L^{-1} in GAPDH and ubiquitin, respectively. When using S_2 values as a calibrator, the similarly estimated LC_{50} values were 457 and 499 mg L^{-1}, respectively (Data was not shown). Those values were roughly corresponding with the data in Bajda et al. (2017).

Table S1. Primers used for amplification of *nad1* and *nad5* from *T. urticae* strains NPS and NPR

Target gene	Primer name	Sequence (5′–3′)	T_m (°C)	Amplicon size
NAD1	nad1_F	TTGCCCATCACTTTCATTTTT	59.4	1559 bp
	nad1_R	CCCGATAAAAGCGGATCCTA	61.3	
NAD5	nad5_F1	ATAGCTGCCCCAACTCCAAT	60.9	890 bp
	nad5_R1	GAAATCTTTTCCATCCATAATTCA	58.4	
	nad5_F2	TGTGTCAATTGTTGTTTTTAATGTTTT	60.0	1713 bp
	nad5_R2	AGGGGGAACCCCTATCTTTA	58.8	

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Table S2. Primer sets for PSST mutation frequency analysis

Primer	Forward (5′–3′)	*E* [a]
Resistance allele-specific primer set		
tu_PSST_222F	TGGAGAGTTTGCTATGGCTCGAATG	1.87
tu_PSST330T_357R	TTCTAAATCATAACGAGGAGCAGCTATTC	
Common primer set for internal reference		
tu_PSST_308F	GTTGTGCCGTTGAAATGATGCAC	R: 1.98
tu_PSST_419R	GTACCAGCGACAATCATGACATCAG	S: 1.90

[a] Amplification efficiency; data from Maeoka et al. (2020); “R” and “S” indicate *E* for DNA samples of NPR and NPS, respectively

Table S3. Primer sets for qPCR of 12 CYP genes upregulated in NPR in RNA-seq

Genes	Gene ID	Forward (5′ – 3′)	Reverse (5′ – 3′)	E_{cDNA}	E_{gDNA}
CYP385C1	*tetur26g01470*	TCTGGTTGGAACTACATTCGCT	AGCTTGTAAACCATCGTATAGACCA	2.01	NA
CYP389C1	*tetur34g00510*	CATGGTACGGCAAAAGTTCTGGAAG	GGATCACGGTGAGTGGAGGAA	2.02	1.93
CYP389C11	*tetur05g06570*	GTTGAACCCTTCTGTTGCTG	TAACCAAAAGGAATCCAAGCTG	2.00	1.98
CYP389C12	*tetur05g06560*	GGAAGTATTCCACTCAAAGCTGAAG	CAGTACAAGCTGCTAATTGTTGGAG	2.12	1.99
CYP392A3	*tetur07g06460*	AGCTTTTCAAACACCAGAATGCGA	CGGCAACGGTCTCTGATCCT	2.03	1.91
CYP392A14	*tetur08g07950*	TGATAGACACCGAGGGAAAC	CTCTGAACCAGCACCGAA	NA	1.93
CYP392A16	*tetur06g04520*	GGTAAACCTGCCGATTTTGA	ACTACACCGGCGAAGCTAAA	1.96	1.89
CYP392B1	*tetur20g03200*	GCCGCTACGATTGTTCCAG	AGGCTTGACTGCTTTGGTTC	2.00	1.90
CYP392D2	*tetur03g04990*	AAACTTGGAGGCAGCAAAGA	TCGCAACACTCAATCCATTC	2.13	1.91
CYP392D4	*tetur03g05010*	CAAAGGACGCTTTTGTCGT	GACTTGACAACCTTAGAACCATC	2.00	1.90
CYP392E4	*tetur27g02598*	CTGGTGAACGGTGGAAGGAAC	CGAAGCATACCATTGGCTTGGA	2.00	1.90
CYP392E8	*tetur27g00350*	ATGCCTACAAACAGATGACGGAGTT	GCCTCCAAGGTTCACCAGTCA	2.05	1.98
GAPDH	*tetur25g00250*	GCACCAAGTGCTAAAGCATGGAG	GAACTGGAACACGGAAAGCCATAC	1.94	1.88
Ubiquitin	*tetur03g06910*	GTCTCCGTGGTGGAATGC	TTGGATTTTGGCTTTCACG	1.98	1.96

E_{cDNA}: Amplification efficiency with cDNA, E_{gDNA}: Amplification efficiency with genomic DNA, NA: Not applicable because of the low expression level (*CYP392A14*, E_{cDNA}) and presence of intron (*CYP385C1*, E_{gDNA}).

Table S4. Primer used for Sanger sequencing

Gene/Vector	Primers	5′ – 3′
CYP392A3	CYP392A3fusion-F	GTGGTGGAATTCTGCCAAAATGTTGCTTCTTGATTACTTTAGTGT
	CYP392A3fusion-R	CTGTGCTGGATATCTTTATACTCTTTGGAAGATTAAATCTGG
	CYP392A3-forward	ATGTTGCTTCTTGATTACTTTAGTGT
	CYP392A3-reverse	TTATACTCTTTGGAAGATTAAATCTGG
	CYP392A3-692F	AAGTGGCGATTGATGAAAGTCTG
	CYP392A3-938R	AGTTGATCTTTCCGCTTAGACTG
pAc5.1/V5-HisA	Ac5-seq-F	ACACAAAGCCGCTCCATCAG
	Ac5-seq-R	TAGAAGGVAVAGTCGAGG
PSST	PSST-73F	GGAGCCGTTGCTTCTATTTCAACC
	PSST-664R	CAGTGGCTTGTCGCTTAATCCTC

Table S5. Statistics of RNA-seq reads of NPR and NPS strains

Strain	Treatment	Biological replicate	No. of reads		Rate of clean reads
			Raw	Clean	
NPR	Water	Rep1	30,861,954	28,368,112	91.92%
		Rep2	27,273,852	24,980,852	91.59%
		Rep3	30,201,616	27,785,640	92.00%
	Pyridaben	Rep1	31,028,030	28,325,300	91.29%
		Rep2	26,760,276	24,584,322	91.87%
		Rep3	24,414,942	22,389,594	91.70%
NPS	Water	Rep1	25,641,614	23,300,040	90.87%
		Rep2	28,035,392	25,797,942	92.02%
		Rep3	26,524,690	24,187,240	91.19%
	Pyridaben	Rep1	25,921,118	23,709,410	91.47%
		Rep2	29,352,400	26,944,552	91.80%
		Rep3	24,750,876	22,788,432	92.07%

Table S6. Statistics of reference transcriptome assembly

	transcripts	genes
No. of sequences	39,245	28,406
Total size (bp)	54,954,726	33,087,068
Average length (bp)	1,400	1,164
Maximum length (bp)	20,873	20,873
N50 (bp)	2,651	2,473
E90N50 (bp)	3,132	3,318
Number of predicted CDS	17,161	10,674
Number of blastn hits with gene set 20130928	21,598	12,965
Number of blastx hits with NCBI-nr	19,768	12,263

The reference transcriptome assembly was generated by de novo assembly using the six RNA-seq samples of NPR strain. Assembled contigs (transcripts) were clustered into genes (Trinity component) based on sequence similarity by Trinity software. Only longest contig per gene was used for calculating the statistics of the genes. E90N50 is an N50 value calculated using highly expressed contigs accounting for 90% of total expression, which is a more effective metrics for evaluating quality of the transcriptome assembly than the N50.

Table S7. The number of mapped RNA-seq reads with and without H110R mutation in PSST gene

Strain	Treatment	Biological replicate	No. of mapped reads		Rate of resistant reads
			CAC (H) (Susceptible)	CGC (R) (Resistant)	
NPR	Water	Rep1	0	147	100%
		Rep2	0	109	100%
		Rep3	0	174	100%
	Pyridaben	Rep1	0	156	100%
		Rep2	0	120	100%
		Rep3	0	103	100%
NPS	Water	Rep1	115	0	0%
		Rep2	153	0	0%
		Rep3	114	0	0%
	Pyridaben	Rep1	90	0	0%
		Rep2	110	0	0%
		Rep3	111	0	0%

Table S8. Recombination rate of PSST and CYP392A3 loci in F2 males in uncoupling experiments.

Parental crosses	No. of males detected [a]				Recombination rate
(♀ × ♂)	R-INS2+	H-INS2−	R-INS2−	H-INS2+	(%)
SS_1 × RR_1	34	43	0	2	2.5
SS_2 × RR_2	36	37	1	1	2.7

[a] R and H indicate H110R and H110, respectively, in *PSST*, and INS2+ and INS2− show c.1242_1243insAATATTACC (p.E414_N415insNIT; INS2+ type) and c.1246_1247 (INS2− type) in *CYP392A3*.