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The generalist herbivore *Tetranychus urticae* (Koch) adapts to novel plant hosts through rapid evolution of metabolic resistance

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Abstract

Genetic adaptation, occurring over long evolutionary time, enables host-specialized herbivores to develop novel resistance traits and to counteract the defenses of a narrow range of host plants. In contrast, physiological acclimation, leading to the suppression and/or detoxification of host defenses is hypothesized to enable generalists to shift between plant hosts. Here, we examined the long-term response of an extreme generalist, the two-spotted spider mite, *Tetranychus urticae* Koch (TSSM), to the shift to the non-preferred and novel host plant *Arabidopsis thaliana*. We identified the key requirement of two tiers of cytochrome P450 monooxygenases for TSSM adaptation to *Arabidopsis*: general xenobiotic-responsive P450s that have a limited contribution to mite adaptation to *Arabidopsis* and adaptation-associated P450s that efficiently counteract *Arabidopsis* defenses, illustrating that in about 25 generations of TSSM selection on *Arabidopsis* plants mites evolved metabolic resistances characteristic of both generalist and specialist herbivores.

Keywords: *Arabidopsis*, host shift, biochemical adaptation, plant allelochemicals, metabolic resistance, suppression of plant defenses, xenobiotic responsiveness, detoxification, *NADPH-cytochrome P450 reductase*, RNAi, plant-herbivore interaction

Introduction

During millions of years of co-evolution with their host plants, herbivores have developed two main strategies to counteract plant resistance traits. Specialist herbivores have evolved highly efficient adaptation mechanisms against a limited set of host defenses, including: modified feeding behavior (Helmus and Dussourd, 2005), suppression of plant defenses (Zhao et al., 2015), reduced xenobiotic target site sensitivity (Dobler et al., 2012), sequestration (Beran et al., 2018) and detoxification of plant toxins (Ratzka et al., 2002). In contrast, generalist herbivores evolved an innate ability to feed on a broad range of host plants that display a wide array of resistance traits (Despres et al., 2007; Barrett and Heil, 2012; Heidel-Fischer and Vogel, 2015). Attenuation of plant responses induced by herbivore feeding, consistent with the suppression of host defenses, have been described for many generalists (Zarate et al., 2007; Kant et al., 2008; Musser et al., 2012; Wu et al., 2012). Consistently, effectors that modulate plant defenses have been identified in secretions of a number of generalist herbivores belonging to different feeding guilds (Musser et al., 2002; Hogenhout and Bos, 2011; Wu et al., 2012; Bass et al., 2013; Jonckheere et al., 2016; Kaloshian and Walling, 2016; Basu et al., 2018). Their mode of action is largely unknown but, to be effective against many host plants, it is assumed that they either target conserved compounds or pathways associated with plant defense, or that they collectively have a very broad spectrum activity with only a specific subset being effective against any particular host. Another mechanism of host-adaptation is metabolic resistance whereby herbivores effectively detoxify ingested plant toxins. In specialists, metabolic resistance is based on a limited number of detoxification enzymes that have high specificity and efficiency for a given plant toxin (Ratzka et al., 2002; Li et al., 2003; Wittstock et al., 2004; Mao et al., 2006; Gloss et al., 2014; Heidel-Fischer et al., 2019). Genes encoding these enzymes are usually expressed constitutively at high level and can be further induced in the presence of plant toxin. In contrast, it is assumed that generalist herbivores rely on ubiquitous classes of detoxification enzymes (e.g. carboxyl/cholinesterases (CCEs), cytochrome P450 monooxygenases (CYP450s), glutathione S-transferases (GSTs), UDP-glycosyltransferases (UGTs), and ABC transporters (ABCs)) that were shown to accept structurally diverse substrates which they metabolize with low levels of activity (Li et al., 2004; Halon et al., 2015; Shi et al., 2018; Snoeck et al., 2019). Genes encoding general detoxification enzymes underwent extensive amplification and neofunctionalization, and are transcriptionally responsive to a wide range of xenobiotics (Govind et al., 2010; Grbic et al., 2011; Zhurov et al., 2014; Wybouw et al., 2015; Muller et al., 2017; Schweizer et al., 2017). Thus, it is hypothesized that attenuation of plant defenses combined with the expanded and functionally versatile detoxification capabilities enable generalist herbivores to cope with diverse allelochemicals and to feed on many host plants.

Tetranychus urticae Koch (TSSM) is a striking example of generalist herbivore that feeds on more than 1,100 plant species from over 100 families (Migeon and Dorkeld, 2006-2019). Such a wide host range indicates that TSSM can counteract a great diversity of plant resistance traits. However, individual TSSM populations do not perform equally well on all potential host plants. Instead, TSSM has the outstanding ability to adapt to new hosts, in just 20 to 25 generations (Gould, 1979; Fry, 1989; Magalhaes et al., 2007; Wybouw et al., 2015). The mechanism of this extremely rapid host adaptability is not known. The analysis of plant transcriptional changes following the host shift revealed that some TSSM populations can suppress plant induced responses (Kant et al., 2008; Wybouw et al., 2015). At the same time, TSSM massively reprograms its detoxification capacity (Dermauw et al., 2013; Zhurov et al., 2014; Wybouw et al., 2015) and the complement of its salivary secretions (Jonckheere et al., 2016; Villarroel et al., 2016; Jonckheere et al., 2018). However, no functional evidence explains whether these changes contribute to TSSM host adaptation or if they merely reflect stress

86 responses or a different feeding physiology due to the host shift. Thus, it remains unclear
87 whether adaptation to the new host plant by *T. urticae* requires manipulation of host plant
88 defenses, detoxification of host allelochemicals or both, and if the initial xenobiotic responses
89 are sufficient for TSSM adaptation to a new host, or if changes over a longer period of time are
90 required for the evolution of TSSM host-adaptation.
91 *Arabidopsis thaliana* is a non-preferred host for the TSSM London reference population
92 that is reared on beans – *Phaseolus vulgaris* (Zhurov et al., 2014). In an experimental
93 evolutionary setup, we adapted the TSSM London ancestral population to *Arabidopsis.* Taking
94 advantage of well characterized *Arabidopsis* induced defenses against mite herbivory (Zhurov et
95 al., 2014), we show that *Arabidopsis*-adapted TSSM do not suppress them. Using manipulative
96 pharmacological and reverse genetics experiments that independently suppressed the activities
97 of families of detoxification enzymes we provide, for the first time, *in vivo* functional evidence
98 that *T. urticae* requires two tiers of P450 enzyme activities for host adaptation. We show that
99 P450s responsive to the initial encounter of the new host have a small contribution to mite
100 adaptation to *Arabidopsis*. Instead, a second tier of P450 activity, specific to *Arabidopsis*-
101 adapted mites, is the major contributor to mite adaptation. Our data demonstrate that the
102 reprogramming of the mite detoxification system occurred over approximately 25 generations
103 of TSSM selection on *Arabidopsis* resulting in metabolic resistance with features characterized in
104 both generalist and specialist herbivores. Our results show that the detoxification mechanisms
105 involved in herbivore adaptation to novel host plants can evolve extremely rapidly and are key
106 to TSSM adaptation to *Arabidopsis*.
107
108 **Results and discussion**
109
110 **TSSM feeding induces JA-regulated *Arabidopsis* defense compounds**
111 Jasmonic acid (JA) and its bioactive conjugate jasmonoyl-isoleucine (JA-Ile) were shown to be
112 required for *Arabidopsis* defenses against TSSM herbivory (Zhurov et al., 2014). To determine if
113 JA-induced processes are sufficient for *Arabidopsis* resistance against TSSM, we sprayed
114 *Arabidopsis* plants with methyl jasmonate (MeJA) and determined the effect of induced JA-
115 responses on mite performance. Mite fecundity dramatically decreased (5-fold) on MeJA
116 treated Col-0 plants, Figure 1A, indicating that JA-induced defenses are major components of
117 *Arabidopsis* resistance against mite herbivory. JA responsiveness is mediated by COI1 as well as
118 the MYC2, MYC3 and MYC4 (MYC2,3,4) transcriptional activators that induce the expression of a
119 wide spectrum of JA-regulated genes, Figure 1B and (Devoto et al., 2005; Fernandez-Calvo et al.,
120 2011). The *myc2,3,4* mutant plants were extremely susceptible to *T. urticae*, Figure 1A. Among
121 MYC2,3,4-regulated genes are *CYP79B2* and *CYP79B3* that are required for the synthesis of Trp-
122 derived secondary metabolites, Figure 1A and (Hull et al., 2000; Mikkelsen et al., 2000;
123 Schweizer et al., 2013). Trp-derived secondary metabolites include several known
124 allelochemicals (indole glucosinolates, camalexin and cyanogenic glucosides) directly
125 synthesized from Trp and callose whose synthesis is indirectly regulated through this pathway
126 (Bednarek, 2012; Rajniak et al., 2015). Of these compounds, indole glucosinolates (IGs) were
127 implicated in deterrence of aphid (Kim and Jander, 2007; Kim et al., 2008), whiteflies (Elbaz et
128 al., 2012), TSSM (Zhurov et al., 2014) and leaf mining drosophilid (Whiteman et al., 2011;
129 Whiteman et al., 2012) herbivory. The modest increase in TSSM fecundity when mites fed on
130 *cyp79b2 cyp79b3* plants, Figure 1A, indicates that Trp-dependent defenses have a relatively
131 minor contribution to *Arabidopsis* defenses against TSSM. These data further indicate that
132 MYC2,3,4 induce synthesis of additional *CYP79B2 CYP79B3*-independent *Arabidopsis* defense
133 compounds that prominently contribute to *Arabidopsis* resistance to TSSM. The identity of these

compounds is at present not known. Thus, upon TSSM herbivory, *Arabidopsis* plants induce synthesis of JA-regulated defense compounds against TSSM, including Trp-derived secondary metabolites and, *CYP79B2 CYP79B3*-independent molecules, Figure 1B.

Mites can adapt to a complex array of *Arabidopsis* defenses

Because of the complexity of *Arabidopsis* induced defenses against TSSM herbivory, how could a mite population adapted to a specific host plant switch to *Arabidopsis*? In an experimental evolutionary setup, the reference London strain, an ancestral TSSM population reared on bean and highly susceptible to *Arabidopsis* defenses (Zhurov et al., 2014), was transferred and continuously maintained on *Arabidopsis* plants for 18 months (≥25 generations), Figure 2A. We initially infested *Arabidopsis* plants with approximately 1,000 fertilized female mites in a triplicated experiment on two *Arabidopsis* genotypes: a) Col-0 wild-type plants, and b) *cyp79b2 cyp79b3* mutant plants that lack Trp-derived defenses but express the remaining of JA-regulated defenses, Figures 1B and 2A. The performance of the ancestral and selected mite populations was subsequently quantified by counting the number of eggs, larvae, nymphs and adults derived from 20 adult females in 7 days. Performance was measured in two experimental regimes: a) direct transfer, where the initial mites were moved from their corresponding rearing plant hosts directly to the experimental plants, and b) indirect transfer, that included mite maintenance on bean plants for two generations prior to their transfer to the experimental plants. The performance of the ancestral and selected mite populations on bean, or *Arabidopsis cyp79b2 cyp79b3* and Col-0 plants had similar patterns in both direct (Supplemental Figure 1) and indirect (Figure 2B) transfer regimes, indicative of genetic adaptation that is independent of maternal and environmental physiological effects.

On bean plants, Figure 2B top, *Arabidopsis*-selected mite populations had slight but significantly lower performance relative to the ancestral population, indicating that TSSMs can evolve the ability to exploit different hosts without major reduction in their performance on the ancestral plants. On *cyp79b2 cyp79b3* plants, Figure 2B middle, *Arabidopsis*-selected mite populations performed significantly better than the ancestral population, suggesting that they are able to overcome *CYP79B2 CYP79B3*-independent *Arabidopsis* defenses to a similar extent. On Col-0 plants, Figure 2B bottom, only mites selected on Col-0 had increased performance over the ancestral population, showing that Col-0 selected mites were able to adapt to *CYP79B2 CYP79B3*-dependent defenses. However, mites selected on *cyp79b2 cyp79b3* plants and the ancestral mite population that were not exposed to *CYP79B2 CYP79B3*-dependent *Arabidopsis* defenses were susceptible to these defenses and had similar and low performances when they fed on Col-0 plants.

We have arbitrarily chosen populations #3 of *cyp79b2 cyp79b3* and Col-0 selected mites for further studies that were performed in indirect transfer regimes. The analysis of additional mite fitness parameters, fecundity and *Arabidopsis* leaf damage caused by mite feeding (Figure 2C and D, respectively) confirmed the adaptation status of these mite populations (from now on referred to as *cyp*-a (for #3 *cyp79b2 cyp79b3* selected mite population), Col-a (for #3 Col-0 selected mite population) and bean-a (for the ancestral London TSSM population)). Thus, our data demonstrate that TSSM can adapt to novel plant hosts with a complex array of defenses. Moreover, mite adaptation to *CYP79B2 CYP79B3*-independent *Arabidopsis* defenses (present in both types of *Arabidopsis*-selected mite populations) can be uncoupled from mite adaptation to Trp-derived defenses (present only in mites selected on Col-0 plants).

182 ***Arabidopsis*-adapted mites do not suppress *Arabidopsis* defenses**
183 Suppression of induced plant defenses was proposed to be the common and most efficient
184 mechanism of TSSM adaptation to new host plants (Jonckheere et al., 2016; Villarroel et al.,
185 2016; Blaazer et al., 2018; Jonckheere et al., 2018). Indeed, interference with either JA-
186 biosynthesis or its signalling appears to be an effective way to attenuate a whole range of
187 *Arabidopsis* defense compounds that restrict TSSM herbivory (Figure 1). Furthermore, the
188 ancestral London strain previously gave rise to tomato-adapted populations that, in the process,
189 gained the ability to suppress tomato JA-regulated responses (Wybouw et al., 2015), indicating
190 that London mites are able to evolve the defense suppression trait.
191 If *Arabidopsis*-adapted mites suppress *Arabidopsis* defenses, then it is expected that
192 they elicit attenuated *Arabidopsis* responses relative to those triggered by the ancestral bean-a
193 mites. The previous transcriptome and metabolome analysis of *Arabidopsis* responses to TSSM
194 herbivory established that the expression of the *AOS, MYC2, CYP79B2* and *CYP79B3* genes
195 (labeled in red in Figure 1B) and the abundance of JA, JA-Ile and indol-3-ylmethylglucosinolate
196 (I3M) metabolites (labeled in green in Figure 1B) are reliable markers of *Arabidopsis* induced
197 defenses, at 24 h following mite infestation (Zhurov et al., 2014). Thus, to investigate whether
198 mite adaptation to *Arabidopsis* relies on the attenuation of JA-induced defenses, we determined
199 the levels of defense marker transcripts (RT-qPCR) and metabolites (liquid chromatography–
200 mass spectrometry, LC-MS) in Col-0 plants that were challenged with bean-a, *cyp*-a or Col-a
201 mites, after 24 h of mite herbivory. Contrary to what would be expected if plant defenses were
202 attenuated, *cyp*-a and Col-a mites induced all JA-regulated marker genes to similar or higher
203 levels relative to non-adapted bean-a mites, Figure 3A. Likewise, JA, JA-Ile and I3M metabolites
204 accumulated at comparable levels in bean-a, *cyp*-a and Col-a challenged Col-0 plants (Figure 3B),
205 indicating that *Arabidopsis* defenses are augmented and not attenuated by mite adaptation. In
206 addition, one of the hallmarks of defense-suppressing TSSM populations is the similarity of their
207 performance on wild-type and defense-deficient host plants (Kant et al., 2008). However,
208 *Arabidopsis*-adapted mites, like the ancestral population, performed better on *cyp79b2 cyp79b3*
209 than on Col-0 plants, Figure 2C and D, indicating that *Arabidopsis*-adapted mites do not
210 manipulate the onset of *Arabidopsis* defenses. Cumulatively, these data demonstrate that
211 adaptation of mites to *Arabidopsis* is not based on the suppression of host defenses.
212
213 ***Arabidopsis*-adapted mites are responsive to *Arabidopsis* xenobiotics**
214 Metabolic resistance is another mechanism of herbivore adaptation to xenobiotic challenges
215 previously implicated in the evolution of TSSM resistance to pesticides (Van Leeuwen and
216 Dermauw, 2016) and mite adaptation to several plant hosts (Agrawal et al., 2002; Wybouw et al.,
217 2012; Wybouw et al., 2014; Wybouw et al., 2015). These previous studies established that
218 constitutive overexpression and/or increased expression of genes encoding xenobiotic-
219 metabolizing enzymes upon exposure to a novel host (transcriptional plasticity) are correlated
220 with TSSM's increased detoxification potential and resistance to pesticides or host
221 allelochemicals. Moreover, the requirement for rapid transcriptional plasticity has been
222 demonstrated in the adaptation of the generalist *M. persicae* to a new host plant (Mathers et al.,
223 2017).
224 We have previously identified forty genes, whose expression levels correlated with
225 increasing levels of *Arabidopsis* defenses (Zhurov et al., 2014). They primarily encoded
226 cytochrome P450 monooxygenases (P450s) and UDP-glycosyltransferase (UGTs) detoxifying
227 enzymes. We have arbitrarily chosen three genes within each class *(CYP392A1, CYP392A16,*
228 *CYP392D8, UGT201A2v2, UGT204B1* and *UGT204A5)* and tracked their expression (RT-qPCR) in
229 bean-a, *cyp*-a and Col-a mites feeding on bean, *cyp79b2 cyp79b3* and Col-0 *Arabidopsis* plants.

230 On the ancestral bean plants, all marker transcripts were at similar low levels in both bean- and
231 *Arabidopsis*-adapted mites, Figure 4, indicating that none of the tested genes underwent
232 constitutive upregulation during mite adaptation to *Arabidopsis*. Consistent with the previous
233 report (Zhurov et al., 2014), transfer of bean-a mites to *cyp79b2 cyp79b3* or Col-0 plants
234 resulted in induced expression of all tested *CYP* and *UGT* genes. Their expression was also
235 induced in *cyp*-a and Col-a mites when they were shifted from bean to *cyp79b2 cyp79b3* or Col-0
236 plants. Even though there was variability in the relative expression of *CYP* and *UGT* genes in
237 these mites, their levels were either comparable or lower than in bean-a mites, Figure 4. Thus,
238 neither constitutive expression nor inducibility of tested *CYP* and *UGT* genes increased in
239 *Arabidopsis*-adapted mites, demonstrating that: a) *Arabidopsis*-adapted mites retained
240 responsiveness to *Arabidopsis* xenobiotics, and b) mite genes initially induced by the shift from
241 bean to *Arabidopsis* plants do not associate with TSSM long-term adaptation to *Arabidopsis*.
242
243 **Metabolic resistance underlies TSSM adaptation to *Arabidopsis***
244 To identify changes in the detoxification potential associated with mite adaptation to
245 *Arabidopsis*, we determined global enzymatic activity for three main protein families - esterases,
246 glutathione-S-transferases (GSTs) and P450s - in ancestral and adapted mite populations upon
247 feeding on bean and *Arabidopsis* plants. Overall, activities of all three enzymatic classes were
248 responsive to the host plant challenge: they were lowest when mites fed on the ancestral bean
249 plants and they progressively increased with the complexity of *Arabidopsis* defenses, Figure 5A,
250 D and Supplemental Figure 2. However, of the three classes of detoxification enzymes, the
251 detoxification potential of P450s is the only one that was associated with TSSM adaptation to
252 *Arabidopsis*. P450 activity was consistently higher in Col-a mites across all plant hosts, indicating
253 that both constitutive and inducible levels of P450 activity increased in Col-a mites, Figure 5D
254 (three-way ANOVA; plant host: mite strain interaction $F = 103.93$, $p = 5.451e-16$, followed by
255 Tukey's HSD test, $p < 0.01$, $n = 4$).
256 To further test the requirement of esterase, GST and P450 activities for TSSM
257 adaptation to *Arabidopsis*, we examined whether the separate enzymatic classes may be
258 correlated with the mite's ability to use *Arabidopsis* as a host. If a particular class of enzymes is
259 required for mite adaptation to *Arabidopsis*, then the reduction of its activity is expected to
260 restore the susceptibility of *Arabidopsis*-adapted mites to *Arabidopsis* defense compounds. We
261 used S,S,S tributyl-phosphorotrithioate (DEF, an inhibitor of esterase activity), diethyl maleate
262 (DEM, an inhibitor of GST activity), and piperonyl butoxide (PBO) and trichlorophenylpropynyl
263 ether (TCPPE) (inhibitors of P450 activity) to inhibit the activity of the indicated enzymatic
264 classes. In preliminary experiments, concentrations of 2000 mg/L (DEF), 100 mg/L (DEM), 1000
265 mg/L (PBO), and 1500 mg/L (TCPPE) were identified as sublethal but nevertheless capable of
266 significantly reducing the matching enzymes in Col-a mites feeding on Col-0 plants, Figure 5B, E
267 and G, and Supplemental Figure 2. Note that inhibitors do not affect all enzymes equally within
268 the targeted enzymatic class (Feyereisen, 2014). Therefore, the lack of inhibitory effect on mite
269 performance is not a strong evidence against the involvement of a particular enzymatic class in
270 mite host adaptation. Conversely, the significant decrease of mite performance on a new host
271 plant upon the application of a given inhibitor strongly supports the requirement of enzyme(s)
272 within the matching class for mite host adaptation.
273 The application of DEF reduced esterase activities by 32% but had no effect on the
274 fitness of Col-a mites when feeding on either Col-0 or *cyp79b2 cyp79b3* plants, Supplemental
275 Figure 2, indicating that esterases may not be required for mite adaptation to *Arabidopsis*. The
276 application of DEM decreased the GST enzymatic activity by 43%, Figure 5B. Inhibition of GST
277 activity had a minor but significant effect on the fecundity of Col-a mites, suggesting that GST

activity is required for the high Col-a performance on *Arabidopsis*, Figure 5C. Isothiocyanates are the major *Arabidopsis* anti-herbivore defense compounds that are detoxified by GSTs in many generalist caterpillars (Wadleigh and Yu, 1988; Schramm et al., 2012; Jeschke et al., 2016) and leaf mining drosophilids (Gloss et al., 2014). However, isothiocyanates are toxic breakdown products of aliphatic glucosinolates, shown to be ineffective against *T. urticae* (Zhurov et al., 2014). Isothiocyanates from Trp-derived indole glucosinolates, a glucosinolate class proposed to be toxic to *T. urticae* (Zhurov et al., 2014), are unstable and are converted to alternative products (Wittstock and Burow, 2010). Whether some of these alternative products confer toxicity against *T. urticae* and are one of the potential substrates for adaptation-associated GSTs remains to be determined.

While esterase and GST inhibitors had no or little effect on TSSM fecundity and fitness, the application of PBO significantly reduced the P450 activity (25%) and dramatically reduced (44%) the fecundity of Col-a mites feeding on Col-a host plants, Figure 5E and F. Since the PBO does not interfere with all P450s equally and also inhibits esterases in some arthropods (Feyereisen, 2014), we applied another structurally unrelated P450 inhibitor, TCPPE, to confirm that the observed effects were due to the decreased P450 activity. TCPPE, like PBO, significantly inhibited P450 enzyme activity in Col-a mites (13%), Figure 5G. It dramatically reduced the fecundity of Col-a mites when they fed on Col-0 (39%) and *cyp79b2 cyp79b3* plants (33%), Figure 5H, suggesting that P450 activity is required for the ability of Col-a mites to counteract *Arabidopsis* defenses.

P450 activity is required for TSSM adaptation to *Arabidopsis*

As P450 inhibitors were applied directly to *Arabidopsis* leaves, there is a possibility that PBO/TCPPE perturbed *Arabidopsis* defense physiology and only secondarily affected mite fecundity. For example, the synthesis of JA metabolites (Heitz et al., 2012; Aubert et al., 2015; Widemann et al., 2015) and Trp-derived secondary metabolites (Hull et al., 2000) occur via pathways that include many plant P450s. Therefore, to further dissect the requirement of mite P450s in TSSM adaptation to *Arabidopsis*, we took advantage of recently validated RNAi gene silencing protocol in TSSM (Suzuki et al., 2017) to silence P450 pathway.

Because the *CYP* gene family is very large in TSSM, it is difficult to characterize the involvement of P450s in mite adaptation to *Arabidopsis* using RNAi (Grbic et al., 2011). To circumvent this hurdle, we instead silenced the expression of *NADPH-cytochrome P450 reductase* (*Tu-CPR*), a co-enzyme required for the catalytic reactions carried out by microsomal P450s (Masters and Okita, 1980). *Tu-CPR* is a single gene in the TSSM genome encoded by the *tetur18g03390* locus (Grbic et al., 2011). It is constitutively expressed in all tissues of adult spider mites, including the midgut epithelial and digestive cells where digestion and detoxification of dietary xenobiotics are expected to take place (Bensoussan et al., 2018), Figure 6A. Application of dsRNA-*Tu-CPR* to Col-a mites resulted in decreased expression of *Tu-CPR,* Figure 6B. Consistent with the Tu-CPR function as an essential component of the P450 enzyme complex, the P450 activity was reduced by 38% in the *Tu-CPR* silenced mites relative to mites exposed to dsRNA-NC (the negative control, dsRNA-NC, is homologous to a non-transcribed genomic region, see Supplemental Table 1 and (Suzuki et al., 2017)), Figure 6C. Strikingly, the silencing of *Tu-CPR* reduced the fecundity of Col-a mites by almost 50% when they were challenged on the *Arabidopsis* host, but had no effect when they infested bean, Figure 6D. Similar phenotypes were obtained upon the application of a second, independent, dsRNA-*Tu-CPR-1* fragment, Supplemental Figure 3, confirming the specificity of the observed phenotypes to a loss of *Tu-CPR* function and the requirement of P450 activity for the adaptation of Col-a mites to *Arabidopsis*.

The RNAi reverse genetics approach combined with the application of P450 inhibitors identified several features of P450 activity. First, the reduced P450 activity, through the application of P450 inhibitors or dsRNA-*Tu-CPR*, did not affect TSSM fecundity when mites fed on bean plants - an ancestral host (Figure 5H and 6D, and Supplemental Figure 3), suggesting that P450 activity is specifically required for mites' ability to use *Arabidopsis* as a host. Second, the experimental treatments reduced global P450 activity but did not eliminate it, Figures 5E, 5G and 6C. Despite the incomplete inhibition of P450 activity, the fitness advantage of Col-a mites over the ancestral population on Col-0 plants was almost completely abolished (Figures 5F, 5H 6D and Supplemental Figure 2), demonstrating that P450 activity is the main contributor to TSSM adaptation to *Arabidopsis*. Third, P450 inhibitors had a limited but significant effect on the fecundity of bean-a ancestral TSSM population when these mites were challenged on the *Arabidopsis* host (Figures 5F and H). As bean-a mites had no prior exposure to *Arabidopsis* plants, the effect of P450 inhibitors on their fecundity uncovers the limited contribution of initially induced P450s to mite fitness on *Arabidopsis.* Since, *Arabidopsis*-adapted mites retained responsiveness to shift from bean to *Arabidopsis* plants, Figure 4, it is expected that these P450s also contribute to the resistance of *Arabidopsis*-adapted mites to *Arabidopsis* defenses. Fourth, the reduced P450 activity profoundly affected the fecundity of Col-a mites when exposed to *Arabidopsis* defenses (Figures 5F and H, and Supplemental Figure 2), indicating that Col-a mites acquired highly efficient P450 enzymatic catalytic activity(ies) against *Arabidopsis* defenses during the adaptation process. At least some of these P450 proteins should be encoded by *CYP* genes with increased basal expression, as increased P450 activity was detected in bean-fed Col-a mites, Figure 5D. Fifth, inhibition of P450 activity affected Col-a mite fecundity when they fed on both Col-0 and *cyp79b2 cyp79b3* plants, Figures 5F and H. This indicates that P450s function to counter CYP79B2 CYP79B3-independent defenses, shown to be major contributors to deterrence of TSSM herbivory, Figure 1.

In summary, we provided robust *in vivo* functional data proving that P450 activity is required for *T. urticae* adaptation to the *Arabidopsis* host plant. In an unbiased analysis, we identified two distinct modes of P450 activities, Figures 4-6. One corresponds to the early responsive *CYP* genes that are induced in both ancestral and *Arabidopsis*-adapted mites upon exposure to *Arabidopsis*. These *CYPs* enable limited adaptation to *Arabidopsis* defenses ((Zhurov et al., 2014) and this study). This is consistent with the canonical detoxification mechanism in generalist herbivores associated with physiological acclimation whereby the exposure to xenobiotics results in the transcriptional induction of genes encoding enzymes capable of metabolizing these defense compounds (Li et al., 2004; Sasabe et al., 2004; Mathers et al., 2017; Shi et al., 2018). Typically, such enzymes recognize a wide range of substrates and have low enzymatic catalytic activity, resulting in a limited contribution to the detoxification of plant defense compounds. The other more significant contribution of P450-mediated metabolic resistance is realized through the action of adaptation-associated P450s (Figures 5, 6 and Supplemental Figure 3). This pattern of metabolic counteraction is reminiscent of strategies deployed by host-specialised herbivores, in which host-adaptation associates with mutations that enhance the enzyme activity (Li et al., 2007; Schuler, 2011) or with the overexpression of detoxification genes (Bass et al., 2013). Therefore, we interpret the adaptation of TSSM populations to *Arabidopsis* as a combination of metabolic resistances characteristic of both generalist and specialist herbivores. Remarkably, the host-shift in TSSM occurs within an ecological timeframe, over 25 generations, enabling individual TSSM populations to expand its host range without losing the ability of feeding on the ancestral host.

374 **Conclusion**
375 The innate ability to perceive and respond to plant defenses in a process of physiological
376 acclimation is assumed to enable generalist herbivores to utilize a wide range of host plants. On
377 the other hand, genetic adaptation, occurring over long evolutionary time scales, is presumed to
378 enable specialist herbivores to evolve novel adaptation traits that efficiently counteract
379 defenses of a narrow range of host plants (Despres et al., 2007; Barrett and Heil, 2012; Heidel-
380 Fischer and Vogel, 2015). *T. urticae* is an extreme generalist that acclimates, like other
381 polyphagous herbivores, to host shift by reprogramming its salivary and detoxification
382 complements (Figure 4 and (Dermauw et al., 2013; Zhurov et al., 2014; Wybouw et al., 2015;
383 Jonckheere et al., 2016; Villarroel et al., 2016; Jonckheere et al., 2018)). However, prolonged
384 exposure (over generations) to initially unfavorable host plants enables *T. urticae* to dramatically
385 increase its performance (Figure 2 and (Gould, 1979; Fry, 1989; Magalhaes et al., 2007; Wybouw
386 et al., 2015)). As demonstrated here for the *Arabidopsis*-adapted mite populations, these mites
387 undergo genetic adaptation to a complex mixture of defenses presented by the new host plant.
388 Even though suppression of plant defenses has been proposed to be the prevailing
389 mechanism of adaptation for generalist herbivores (Jonckheere et al., 2016; Villarroel et al.,
390 2016; Blaazer et al., 2018; Jonckheere et al., 2018), our data show that neither *cyp*-a nor Col-a
391 mite populations attenuate *Arabidopsis* JA-induced defenses. Instead, we provide multiple
392 independent lines of evidence indicating that *Arabidopsis*-adapted mites evolve metabolic
393 resistances characteristic of both generalist and specialist herbivores. Notably, these adaptation
394 mechanisms unfolded over approximately 25 generations, indicating that *T. urticae* has the
395 ability to reset its interaction with the host plant extremely rapidly.
396 The genomic repertoire of detoxification genes in TSSM could be the versatile toolkit
397 enabling that generalist mite to respond to a wide range of plant defenses. If it is so, the
398 comparative genome and transcriptome analysis of *Arabidopsis*-adapted and ancestral TSSM
399 populations may identify genes within the P450 family that are required for host adaptation to
400 *Arabidopsis* and reveal if adaptation-associated P450 activity is achieved by altering gene
401 regulation, copy number or through specific mutation(s). Whether the long-term genetic
402 adaptation to the new host described in this study is exclusive to *T. urticae* or is widespread
403 among generalist herbivores remains unknown. However, understanding the mechanisms
404 involved in generalist adaptation is highly relevant in the context of anthropogenic
405 environmental alterations characterized by habitat fragmentation and loss, climate change and
406 invasion of alien species. These changes are reshaping ecological communities and ecological
407 interactions, and are expected to promote the proliferation of generalist species (DeLucia et al.,
408 2012; Stireman and Singer, 2018).
409
410 **Materials and methods**
411 **Plant growing and mite rearing conditions**
412 Columbia-0 (Col-0) seeds were obtained from the Arabidopsis Biological Resource Center (ABRC,
413 Ohio State University), *cyp79b2 cyp79b3* from B. A. Halkier (University of Copenhagen,
414 Denmark), *myc2 myc3 myc4*, from R. Solano (Universidad Autónoma de Madrid) and bean
415 (*Phaseolus vulgaris*, cultivar California Red Kidney) from Stokes, Thorold, Ontario, Canada.
416 *Arabidopsis* plants were grown under 100 to 150 µmol m^{-2} sec^{-1} cool-white fluorescent light at
417 24°C with a 10-h/14-h (light/dark) photoperiod in controlled growth chambers. The two spotted
418 spider mites (*Tetranychus urticae*), London reference strain (Grbic et al., 2011), were reared on
419 bean plants in growth chambers at 24°C, 60% relative humidity, and with a 16-h/8-h (light/dark)
420 photoperiod for more than 100 generations.
421

422 **Experimental evolution**
423 The London strain, reared on bean plants over the last ten years (referred to as bean-a strain), is
424 an ancestral line used in the experimental evolution. Three independent selection lines were
425 generated by transferring approximately 1,000 randomly chosen adult bean-a females to 3-
426 week-old *cyp79b2 cyp79b3* or Col-0 *Arabidopsis* plants that were replaced biweekly. Mite
427 populations were allowed to propagate for at least 25 generations, generating six selected lines:
428 three independent lines selected on *cyp79b2 cyp79b3* plants, referred to as *cyp*-a lines (#1-#3),
429 and three independent lines selected on Col-0 plants, referred to as Col-a lines (#1-#3). Selected
430 lines were subsequently continuously reared on their corresponding plant hosts. *cyp*-a line (#3)
431 and Col-a line (#3) were used for the follow-up experiments.
432
433 **Mite performance analysis**
434 Twenty 3-day-old adult female mites were transferred to each of the experimental plants (bean,
435 *cyp79b2 cyp79b3* and Col-0) to be evaluated for their performance. Mite performance was
436 measured as total population size seven days post inoculation in direct (mites were moved
437 directly from their rearing hosts to experimental plants) and indirect (mites were reared on
438 bean plants for two generations prior to their transfer to the experimental plants) transfer
439 regimes. The experiment was performed in four biological replicates for each selected line.
440 Differences between selected line population sizes were determined using a one-way ANOVA,
441 followed by Tukey's honestly significant difference (HSD) test. All other experiments were
442 performed with mites that were propagated for two generations on bean plants.
443
444 **Plant damage analysis**
445 Leaf damage of *cyp79b2 cyp79b3* and Col-0 *Arabidopsis* plants upon feeding of bean-a, *cyp*-a
446 and Col-a mite strains was performed as previously described (Zhurov et al., 2014). Briefly, ten
447 adult female mites were placed on the rosette of *Arabidopsis* plants and allowed to feed for 3
448 days before plants were cut at the base of the rosette. The adaxial side of the rosette was
449 scanned using a Canon® CanoScan 8600F model scanner (Canon U.S.A. Inc., Melville, NY, U.S.A)
450 at a resolution of 1200 dpi and a brightness setting of +25. Scanned plants were saved as .jpg
451 files for subsequent analysis in Adobe Photoshop 5 (Adobe Systems, San Jose, CA) as previously
452 described. The experiment was performed using 6 biological replicates/trial, and in three
453 experimental trials. Differences in plant damage between mite strains on different hosts were
454 determined using a three-way ANOVA, using mite strain, plant host and experimental replication
455 as main effects, including interaction terms, followed by Tukey's honestly significant difference
456 (HSD) test. There was a lack of interaction between experimental trials and other main effects,
457 so data across trials were combined for increased statistical power by use of the three-way
458 ANOVA (Brady et al., 2015).
459
460 **Mite fecundity assay**
461 Fecundity of bean-a strain on Col-0, *cyp79b2 cyp79b3* and *myc2 myc3 myc4* as well as bean-a,
462 *cyp*-a and Col-a strains when feeding on *cyp79b2 cyp79b3* or Col-0 *Arabidopsis* plants were
463 performed on detached leaves. Briefly, a petiole of a detached fully-elongated adult leaf was
464 mounted through the parafilm into a small petri plate filled with water. The detached leaf was
465 infested with a single 3-day-old adult female mite whose fecundity was monitored over two or
466 six days. A vented lid was placed on-top of the set up to ensure the mite containment.
467 Inoculated leaves were kept under standard mite rearing conditions and were replaced every
468 second day in the case of 6-day period. The experiment included five to ten biological replicates
469 for each treatment that were repeated in three experimental trials. Differences in fecundity

470 between mite strains on different hosts were determined using a three-way ANOVA, using mite
471 strain, plant host and experimental trial as main effects, including interaction terms, followed by
472 Tukey's honestly significant difference (HSD) test. There was a lack of interaction between
473 experimental trials and other main effects, so data across trials were combined for increased
474 statistical power (Brady et al., 2015). For mite fecundity assay on MeJA-treated Col-0 leaves, the
475 rosette leaves from five-week-old Col-0 plants were sprayed 3 times within 24 h with methyl
476 jasmonate (MeJA) (Sigma-Aldrich, Cat # 392707, 500 µM in ethanol 0.4 % (v/v)), or with ethanol
477 0.4 % (v/v). Subsequently, fully-elongated adult leaves were cut and each petiole was inserted in
478 a PCR tube containing 340 µL of 0.5 mg/mL L-Tryptophan (Sigma-Aldrich, Cat # T0254).
479 Detached leaves were sprayed 3 times within 24 h with MeJA or ethanol 0.4 % solution and
480 were transferred in new PCR tubes containing fresh tryptophan solution. Each detached leaf was
481 then infested with 10 adult female mites (London strain) that were starved overnight. The
482 number of eggs were recorded 24 h after mite addition. The experiment included nine biological
483 replicates and was repeated in two experimental trials. Differences in fecundity were detected
484 by a two-way ANOVA, using treatment and experimental trials as main effects including an
485 interaction term. There was a lack of interaction between experimental trials and treatment, so
486 data across trials were combined for increased statistical power (Brady et al., 2015).
487
488 **Real-Time Quantitative Reverse Transcription-PCR Analysis**
489 For plant marker gene analysis, bean-a, *cyp*-a and Col-a mite strains were collected from their
490 rearing hosts and were propagated for two generations on bean plants after which their
491 corresponding 3-day-old adult female mites were transferred to Col-0 plants. Mites were
492 allowed to feed for 24 h, after which leaves were collected and immediately frozen in liquid
493 nitrogen. These samples were used for the plant marker gene/metabolite analysis. Experiment
494 was replicated in three biological replicates/trial and in three independent trials. For TSSM
495 marker gene analysis, bean-a, *cyp*-a and Col-a mite strains were collected from their rearing
496 hosts and were propagated for two generations on bean plants after which their corresponding
497 3-day-old adult female mites were transferred to bean, *cyp79b2 cyp79b3* or Col-0 plants for 24 h.
498 Samples of 100 mites were collected and immediately frozen in liquid nitrogen. The experiment
499 was replicated in three biological replicates/trial and in three independent trials. Preparation of
500 RNA, cDNA and the real-time quantitative reverse transcription-PCR analysis was performed as
501 previously described (Zhurov et al., 2014). Primer sequences and amplification efficiencies (E)
502 used in qPCR are shown in Supplemental Table 1. *PEROXIN4* (*AT5G25760*), was used as the
503 reference gene for *Arabidopsis* genes and the expression of *RP49* (*tetur18g03590*) was used as
504 the reference for mite genes. Ct values of three technical replicates were averaged to generate a
505 biological replicate Ct value. For plotting, an expression values for each target gene (T) was
506 normalized to the reference gene (R). Normalized relative quantity (NRQ) was calculated as NRQ
507 $= (1 + ER)^{CtR}/(1 + ET)^{CtT}$. NRQs were Log2-transformed for statistical analysis. Mite marker gene
508 data were analyzed by a two-way ANOVA with mite strain and plant host as main effects
509 including an interaction term, followed by Tukey's honestly significant difference (HSD) test
510 (Rieu and Powers, 2009). For plant marker gene analysis, the data were analysed by a two-way
511 ANOVA, using mite strain and experimental trial as main effects including an interaction term,
512 followed by Tukey's honestly significant difference (HSD) test. There was a lack of interaction
513 between experimental trial and mite strain, so data across trials were combined for increased
514 statistical power (Brady et al., 2015). For graphical representation of the expression of plant
515 marker genes, the NRQ data were further normalized to the 'no mite' control sample, which was
516 therefore set to one. Thus, these results represent fold change differences in the expression of
517 marker genes relative to 'no mite' control.

518 **Metabolic Analysis**
519 Plant material used for the RNA isolation toward the expression analysis of the plant marker
520 genes was utilized for the quantification of plant metabolites (JA, JA-Ile and I3M) as described in
521 Zhurov et al., 2014. Differences in metabolite levels were detected by a two-way ANOVA, using
522 mite strain and experimental trial as main effects including an interaction term, followed by
523 Tukey's honestly significant difference (HSD) test. There was a lack of interaction between
524 experimental trial and mite strain, so data across trials were combined for increased statistical
525 power (Brady et al., 2015).
526
527 **Determination of cytochrome P450 monooxygenase (P450), esterase and glutathione-S-**
528 **transferase (GST) activity**
529 To perform the enzymatic activity assays, 3-day-old spider mite females of each Col-a, *cyp*-a and
530 bean-a strains, reared for two generations on bean plants, were placed on bean, *cyp79b2*
531 *cyp79b3* or Col-0 plants. After 24 h, 200 females from each treatment were collected and
532 immediately frozen, to be used for protein extraction and enzymatic assays. Mites were
533 homogenized in 100 mM phosphate buffer, pH 7.6. The concentration of total protein mite
534 extracts was determined using the Quick Start™ Bradford Protein Assay (Quick start Bradford 1x
535 dye reagent, Bio-Rad, Cat# 500-0205), with Bovine Serum Albumin (Sigma-Aldrich, Cat # A7906)
536 as the standard. Twenty, 10 and 10 µg of protein per reaction were used for measuring
537 enzymatic activity of glutathione-S-transferases (GSTs), P450 monooxygenases, and esterases,
538 respectively. The enzymatic activities were assessed spectrophotometrically using 1-chloro-2,4-
539 dinitrobenzene (CDNB) (for GSTs) (Habig and Jakoby, 1981), 7-ethoxy-4-trifluoromethylcoumarin
540 (7-EFC) (for P450s) (Buters et al., 1993), and 4-nitrophenyl acetate (pNPA) (for esterases) (Park
541 et al., 1961), as substrates. Enzymatic activities were determined in four independent samples
542 with three technical replicates per sample. Differences in enzymatic activity between mite
543 strains on different plant hosts were detected using a two-way ANOVA, with mite strain and
544 plant host as main effects including an interaction term, followed by Tukey's honestly significant
545 difference (HSD) test.
546
547 **Application of enzyme inhibitors**
548 Diethyl maleate (DEM, an inhibitor of glutathione S-transferase *(*GST*)* activity), S,S,S tributyl-
549 phosphorotrithioate (DEF, an inhibitor of esterase activity), and piperonyl butoxide (PBO) and
550 trichlorophenylpropynyl ether (TCPPE) (inhibitors of P450 activities) were dissolved in acetone
551 (1:1 by volume) and were subsequently diluted with distilled water to reach desired
552 concentrations. A range of concentrations of DEM (100, 200, 500, 1000 and 2000 mg/L), DEF (10,
553 20, 100, 200 and 500 mg/L), PBO (30, 100, 500, 1000 and 2000 mg/L) and TCPPE (1500 and 2000
554 mg/L) were tested to identify highest sublethal inhibitor concentration (mortality below 10%).
555 Briefly, a batch of five detached bean leaves was sprayed either with a solution containing an
556 inhibitor or a mock solution to the point of run-off. Fifty 1-2-day-old adult bean-a female mites
557 were transferred to the upper (adaxial) side of a treated bean leaf that was placed on wet
558 cotton wool. After 24 h, the number of surviving mites was recorded and mortality was
559 calculated using Abbott's formula (Abbott, 1925).
560
561 For inhibitor bioassays, detached leaves of Col-0 *Arabidopsis* plants were dipped in a solution
562 containing one of the inhibitors (DEF, DEM, PBO, TCPPE) or water and acetone only as control.
563 Upon drying, leaves were infested with 1-2-day-old adult female mites. For fecundity assays,
564 one adult bean-a or Col-a female was placed on the leaf and the number of eggs laid by each
565 female per day was recorded for six days. Treated Col-0 leaves were replaced every other day.

566 Fecundity was determined in three independent experimental trials with ten replicates per trial.
567 For enzymatic assays, each Col-0 leaf was infested with ten 1 to 2-day-old adults Col-a female
568 mites. After 24 h, a pool of 100 spider mites was used to measure GST, esterase and P450
569 activities in DEM-, DEF-, PBO- and TCPPE-treated spider mites, respectively, using the protocol
570 described above. Enzymatic activities were determined in five independent samples with three
571 technical replicates per sample. Differences between means of fecundity and enzymatic
572 activities of control and inhibitor-treated samples were calculated using unpaired Student's t
573 tests (following tests for equal variance).
574
575 ***In situ* hybridization**
576 DIG-labelled probes were produced and the whole mount *in situ* hybridization was performed
577 according to previously published methods (Dearden et al., 2002). Images were collected using a
578 Zeiss AxioCam HRc 412-312 camera mounted on a Zeiss Axioplan II microscope.
579
580 **RNAi of *Tu-CPR***
581 Two non-overlapping fragments (*Tu-CPR*, 645 nt) and (*Tu-CPR-F1*, 564 nt), complementary to the
582 coding region of *Tu-CPR (tetur18g03390*) and dsRNA complementary to a 382 bp non-
583 transcribed intergenic fragment spanning the region 1690614±1690995 of the genomic scaffold
584 12 (a negative control dsRNA, referred to as NC) were synthesized using primers listed in
585 Supplementary Table 1 according to previously described protocol (Suzuki et al., 2017). A BLAST
586 search against the *T. urticae* genome confirmed that dsRNA sequences are unique. dsRNA
587 solutions at concentrations of 500 ng/µL were supplemented with 6% (v/v) blue dye
588 erioglaucine (McCormick, Sparks Glencoe, MD). Newly molted adult female mites were soaked
589 in dsRNA/dye solutions at 20°C for 24 h (Suzuki et al., 2017). Post soaking, mites with visible blue
590 dye in posterior midgut were selected and were transferred in batches of 10 to either detached
591 Col-0 leaves or bean leaf disks. Fecundity was determined in three independent experimental
592 trials with ten replicates/trial as number of eggs deposited by individual female mite on 3rd and
593 4th day post soaking. Mites used for the analysis of *CPR* expression levels were further selected
594 on the basis of visual phenotype (spotless). The RT-qPCR was performed in eight (for dsRNA-*CPR*)
595 and three (for dsRNA-*CPR-1*) experimental replicas, as described above. P450 activity in *CPR*
596 silenced mites was determined in five independent samples with three technical replicates per
597 sample, as described above. Differences in fecundity between dsRNA treated mites was
598 detected using a two-way ANOVA, with mite treatment and experimental trial as main effects
599 including an interaction term, followed by Tukey's honestly significant difference (HSD) test.
600 There was a lack of interaction between experimental trial and mite treatment, so data across
601 trials were combined for increased statistical power (Brady et al., 2015). Enzyme activity and RT-
602 qPCR expression levels were compared between dsRNA treated mites using unpaired Student's t
603 tests (following tests for equal variance).
604
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611

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883
884

885 **Figure Legends**

886 **Figure 1.** *Arabidopsis* induced responses to feeding of the two-spotted spider mite (TSSM)
887 *Tetranychus urticae*. (A) The fecundity of bean-reared TSSM (London strain; ancestral population)
888 upon feeding on +/- MeJA treated Col-0 plants (left) and on Col-0 wild type, *cyp79b2 cyp79b3*
889 mutant plants lacking Trp-derived secondary metabolites, and *myc2 myc3 myc4* mutant plants
890 *(myc2,3,4)* that lack JA-dependent induced responses (right). For fecundity on +/- MeJA treated
891 Col-0 leaves, the number of eggs was counted over 24 h and is presented as mean number of
892 eggs laid by a female mite per day ± SEM; experiment was replicated in nine biological
893 replicates/trial in two independent trials. Asterisk indicates a significant difference between
894 treated and control samples (ANOVA: ***P<0.001). For fecundity on different genotypes (right),
895 the number of eggs was counted over two days and is presented as mean number of eggs laid
896 by a female mite per day ± SEM; experiment was replicated in five biological replicates/trial in
897 three independent trials. Different letters represent significant difference between means
898 (Tukey's HSD test, α = 0.05). (B) A simplified schematic of *Arabidopsis* induced responses to
899 TSSM feeding. Jasmonic acid (JA), its bioactive conjugate jasmonoyl-isoleucine (JA-Ile), and
900 MYC2, MYC3 and MYC4 (MYC2,3,4) transcriptional activators transduce the recognition of TSSM
901 herbivory and induce the expression of JA-regulated genes such as *ALLENE OXIDE SYNTHASE*
902 (*AOS), CYP79B2* and *CYP79B3. AOS* is a JA biosynthetic gene, while *CYP79B2* and *CYP79B3*
903 encode enzymes required for the biosynthesis of Trp-derived secondary metabolites.
904

905 **Figure 2.** Experimental evolution of *Tetranychus urticae* adaptation to *Arabidopsis*. (A) A
906 schematic of the experimental evolution procedure. The ancestral population was reared on
907 beans. Triplicated ancestral populations were selected on *cyp79b2 cyp79b3* and Col-0 plants for
908 at least 25 generations. (B) Performance of ancestral and selected populations on bean (top),
909 *cyp79b2 cyp79b3* (middle) and Col-0 (bottom) plants. Mites from *Arabidopsis*-selected
910 populations were transferred from their respective rearing hosts, *cyp79b2 cyp79b3* and Col-0
911 plants, to beans for two generations prior to testing their performance (an indirect transfer
912 regime). The performance was measured as the size of total population derived from twenty
913 adult female mites and seven days post infestation. Data are represented as mean ± SEM, n=4.
914 Statistics were performed on total population counts. See also Figure S1. (C) Fecundity of the
915 ancestral (bean-a) and populations #3 of *Arabidopsis*-selected mites (*cyp*-a and Col-a) upon
916 feeding on Col-0 and *cyp79b2 cyp79b3* plants. Fecundity was measured over six days and data
917 are presented as mean number of eggs laid by a female mite per day ± SEM; experiment was
918 replicated in ten biological replicates/trial in tree independent trials. (D) Leaf damage of Col-0
919 and *cyp79b2 cyp79b3 Arabidopsis* plants upon herbivory of ancestral (bean-a), and populations
920 #3 of *Arabidopsis*-selected mites (*cyp*-a and Col-a). Data are represented as mean ± SEM;
921 experiment was replicated in six biological replicates/trial in three independent trials. Different
922 letters represent significant differences between means (Tukey's HSD test, α = 0.05).
923

924 **Figure 3.** Induced *Arabidopsis* responses upon feeding of bean-a, *cyp*-a and Col-a mites for 24 h.
925 (A) Expression of *AOS, CYP79B2, CYP79B3* and *MYC2* genes in response to bean-a, *cyp*-a and Col-
926 a mite feeding. Shown are means ± SEM of FCs of expression levels detected by RT-qPCR;
927 experiment was replicated in three biological replicates/trial in three independent trials. Primer
928 sequences and amplification efficiencies (E) used in qPCR are shown in Supplemental Table 1.
929 *PEROXIN4* (*AT5G25760*), was used as the reference gene. (B) Levels of JA and JA-Ile (ng g^{-1} fresh
930 weight), and relative level of the indol-3-ylmethyl glucosinolate (I3M, shown as normalized peak
931 area), in 3-week-old Col-0 plants after herbivory of bean-a, *cyp*-a and Col-a mites for 24 h.
932 Values are means ± SEM; experiment was replicated in three biological replicates/trial in two

933 independent trials. Different letters represent significant differences between means (Tukey's
934 HSD test, α = 0.05).
935
936 **Figure 4.** The effect of mite host-adaptation state and plant hosts on the expression levels of
937 candidate *cytochrome P450 (CYP)* and *UDP-glycosyltransferases (UGT)* mite genes. (A) *CYP392A1*
938 (*tetur07g06410*), *CYP392A16* (*tetur06g04520*) and *CYP392D8* (*tetur03g05070*). (B) *UGT201A2v2*
939 (*tetur02g02480*), *UGT204B1* (*tetur02g09830*) and *UGT204A5* (*tetur05g00090*). Shown are
940 means ± SEM for relative quantity of expression normalized to *RP49* (*tetur18g03590*) reference
941 gene, experiment was replicated in three biological replicates/trial; additional two independent
942 experimental trials had similar results. Primer sequences and amplification efficiencies (E) used
943 in qPCR are shown in Supplemental Table 1. Different letters represent significant differences
944 between means (Tukey's HSD test, α = 0.05).
945
946 **Figure 5.** The requirements of glutathione-S-transferase and cytochrome P450 activities for
947 *Tetranychus urticae* adaptation to *Arabidopsis*. (A, D) The activities of glutathione-S-transferases
948 (A) and cytochrome P450s (D) in bean-a, *cyp*-a and Col-a mites (reared on beans for two
949 generations) feeding on bean, *cyp79b2 cyp79b3* or Col-0 plants. Data are represented as mean ±
950 SEM, n=4. Different letters represent significant differences between means (Tukey's HSD test, α
951 = 0.05). (B, E, G) The activities of glutathione-S-transferases (B) and cytochrome P450s (E, G) in
952 Col-a mites feeding on Col-0 plants after the application of diethyl maleate (DEM, an inhibitor of
953 GST activity; in B), piperonyl butoxide (PBO, an inhibitor of P450 activity; in E) and
954 trichlorophenylpropynyl ether (TCPPE, inhibitor of P450 activity; in G). Data are represented as
955 mean ± SEM, n=5. (C, F) The effects of DEM (C) and PBO (F) treatments on fecundity of bean-a
956 and Col-a mites upon feeding on Col-0 and *cyp79b2 cyp79b3* plants. Fecundity was measured
957 over six days and data are presented as mean number of eggs laid by a female mite per day ±
958 SEM; experiment was replicated in ten biological replicates/trial in three independent trials. (H)
959 The effect of TCPPE treatment on fecundity of bean-a and Col-a mites upon feeding on Col-0,
960 *cyp79b2 cyp79b3* or bean plants. Fecundity was measured over six days and data are presented
961 as mean number of eggs laid by a female mite per day ± SEM; experiment was replicated in ten
962 biological replicates/trial in three independent trials. Asterisks in panels B, C, E-H indicate a
963 significant difference between treated and control samples (unpaired Student's t test: *P <0.05,
964 **P<0.01, ***P<0.001). See also Figure S2.
965
966 **Figure 6.** The requirement of CPR and P450 activity for *Tetranychus urticae* adaptation to
967 *Arabidopsis*. (A) Whole-mount *in-situ* hybridization with anti-sense probe of *Tu-CPR* in *T. urticae*
968 (i) adult female, (ii) adult male, (iii) background control (hybridization with sense probe),
969 enlarged view of panel 'i' showing (iv) epithelium (v) ovaries and (vi and vii) digestive cells of
970 adult female. (B-D) The effects of dsRNA-*Tu-CPR*. (B) Relative level of *Tu-CPR* transcript
971 normalized with *RP49* in dsRNA treated Col-a mites (mean ± SEM, n=8, unpaired Student's t test
972 *** P<0.001). (C) The activity of cytochrome P450s in dsRNA treated Col-a mite upon feeding on
973 Col-0 plants (mean ± SEM, n=5, unpaired Student's t test *** P<0.001). (D) The fecundity of
974 dsRNA treated Col-a mites feeding on Col-0 or bean plants. Fecundity was measured over two
975 days (3 and 4 dpi) and data are presented as mean number of eggs laid by a female mite per day
976 ± SEM; the experiment was replicated in ten (for Col-0 plants) and 4 (for bean plants) biological
977 replicates/trial in three independent trials. Data are represented as mean ± SEM (unpaired
978 Student's t test *** P<0.001). Scale bars in (F): (i-iii) 20µm; (iv-vii) 100µm. See also Figure S3.
979

980 **Supplemental Data**
981 **Supplemental Table 1.** Gene-specific primer sequences used for real-time quantitative RT-PCR.
982
983 **Supplemental Figure 1**. Experimental evolution of *Tetranychus urticae* adaptation to *Arabidopsis*.
984 Performance of ancestral and selected populations on bean (top), *cyp79b2 cyp79b3* (middle)
985 and Col-0 (bottom) plants. Mites were transferred directly from their respective rearing hosts to
986 experimental bean, *cyp79b2 cyp79b3* and Col-0 plants. The performance was measured as the
987 size of total population derived from twenty adult female mites and seven days post infestation.
988 Data are represented as mean ± SEM, n=4. Statistics were performed on total population counts.
989
990 **Supplemental Figure 2**. The requirement of esterase activity for *Tetranychus urticae* adaptation
991 to *Arabidopsis*. (A) The esterase activities in bean-a, *cyp*-a and Col-a mites (reared on beans for
992 two generations) feeding on bean, *cyp79b2 cyp79b3* or Col-0 plants. Data are represented as
993 mean ± SEM, n=4. Different letters represent significant differences between means (Tukey's
994 HSD test, α = 0.05). (B) Esterase activity in Col-a mites feeding on Col-0 plants after the
995 application of S,S,S tributyl-phosphorotrithioate (DEF, an inhibitor of esterase activity). Data are
996 represented as mean ± SEM, n=5. (C) Effects of DEF treatment on fecundity of bean-a and Col-a
997 mites upon feeding on Col-0 and *cyp79b2 cyp79b3* plants. Fecundity was assessed as mean
998 number of eggs laid by a female mite in six days ± SEM; the experiment was replicated three
999 times using 10 biological replicates/trial. Asterisks in panels B, C, and E indicate a significant
1000 difference between treated and control samples (unpaired Student's t test: *P <0.05, **P<0.01).
1001
1002 **Supplemental Figure 3.** RNAi silencing of *Tu-CPR* gene. (A) A schematic of the *Tu-CPR* locus. DNA
1003 sequences used for the generation of dsRNA-*Tu-CPR* are shown in red (fragment CPR, 645 bp),
1004 and green (fragment CPR-1, 564 bp). UTR and coding sequences are shown as light and dark
1005 blue boxes, respectively. (B) The effect of dsRNA-*Tu-CPR*. Relative expression of *Tu-CPR*
1006 normalized with *RP49* in dsRNA treated Col-a mites (mean ± SEM, n=8, Student's t test, ns). (C)
1007 The fecundity of dsRNA treated Col-a mites feeding on Col-0 and bean plants. Fecundity was
1008 measured over two days (3 and 4 dpi) and data are presented as mean number of eggs laid by a
1009 female mite per day ± SEM; the experiment was replicated in ten (for Col-0 plants) and 4 (for
1010 bean plants) biological replicates/trial in three independent trials. Data are represented as mean
1011 ± SEM (Student's t test *** P<0.001).

FIGURE 1

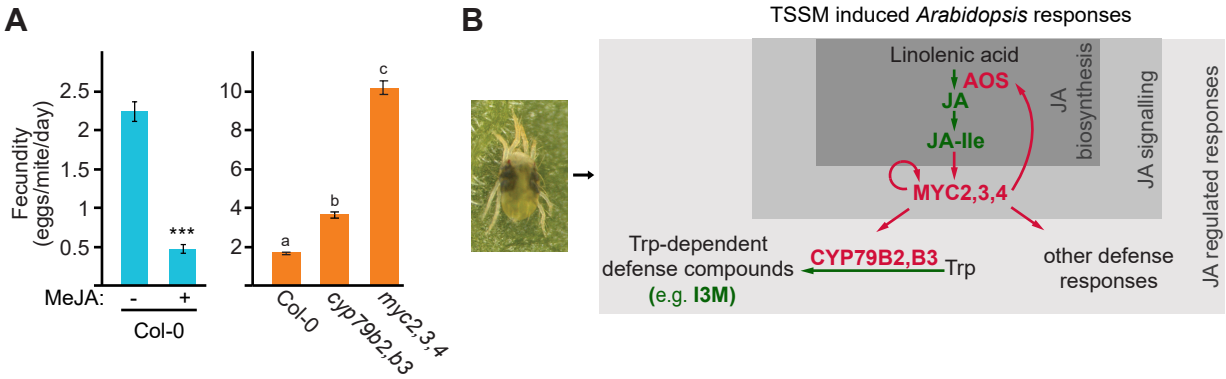

A
Fecundity (eggs/mite/day)
2.5
2
1.5
1
0.5
***
MeJA:
-
+
Col-0
10
8
6
4
2
a
b
c
Col-0
cyp79b2,b3
myc2,3,4
B
TSSM induced Arabidopsis responses
Linolenic acid
AOS
JA
JA-Ile
MYC2,3,4
JA biosynthesis
JA signalling
JA regulated responses
Trp-dependent defense compounds
(e.g. I3M)
CYP79B2,B3
Trp
other defense responses

FIGURE 2

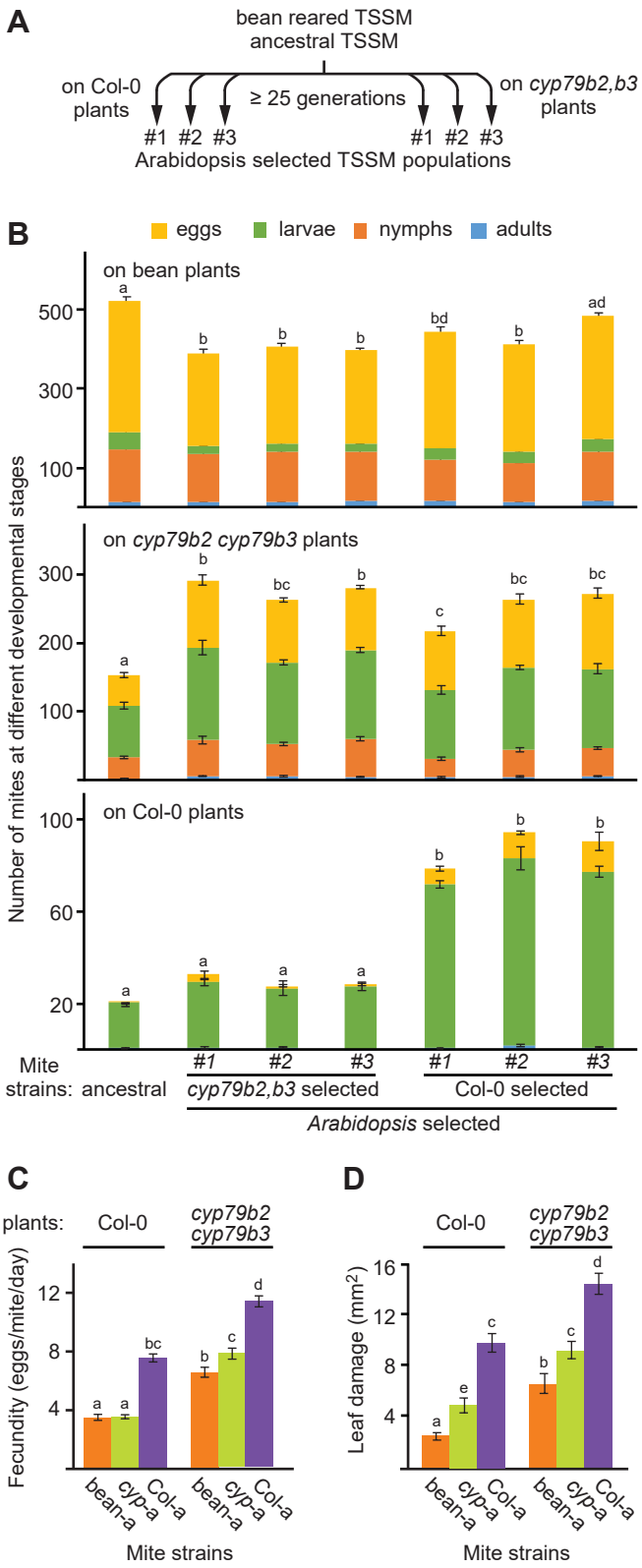

A
bean reared TSSM
ancestral TSSM
on Col-0 plants
≥ 25 generations
on cyp79b2,b3 plants
#1 #2 #3
#1 #2 #3
Arabidopsis selected TSSM populations
B
eggs
larvae
nymphs
adults
on bean plants
on cyp79b2 cyp79b3 plants
on Col-0 plants
Number of mites at different developmental stages
Mite strains: ancestral
#1 #2 #3
cyp79b2,b3 selected
#1 #2 #3
Col-0 selected
Arabidopsis selected
C
plants: Col-0
cyp79b2 cyp79b3
Fecundity (eggs/mite/day)
bean-a
cyp-a
Col-a
Mite strains
D
Col-0
cyp79b2 cyp79b3
Leaf damage (mm2)
bean-a
cyp-a
Col-a
Mite strains

FIGURE 3

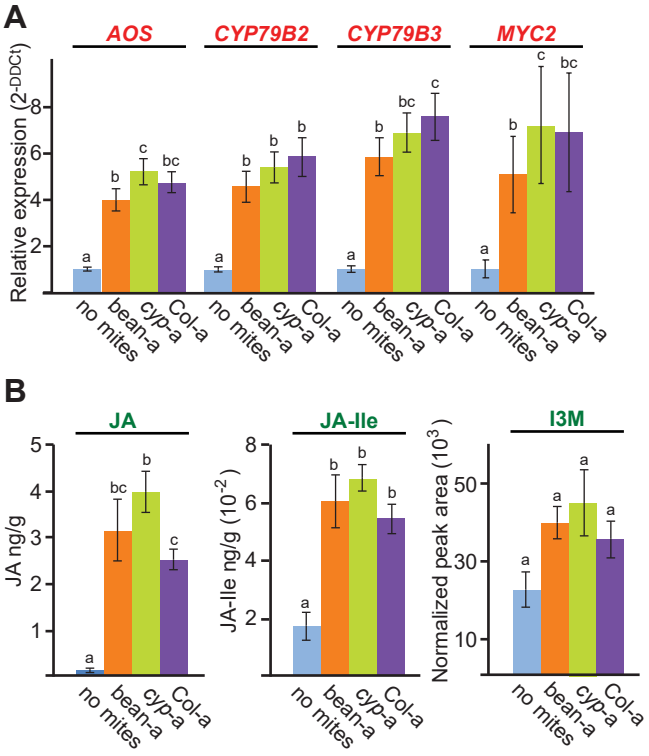

A
AOS
CYP79B2
CYP79B3
MYC2
Relative expression (2-DDCt)
no mites
bean-a
cyp-a
Col-a
B
JA
JA-Ile
I3M
JA ng/g
JA-Ile ng/g (10-2)
Normalized peak area (103)

FIGURE 4

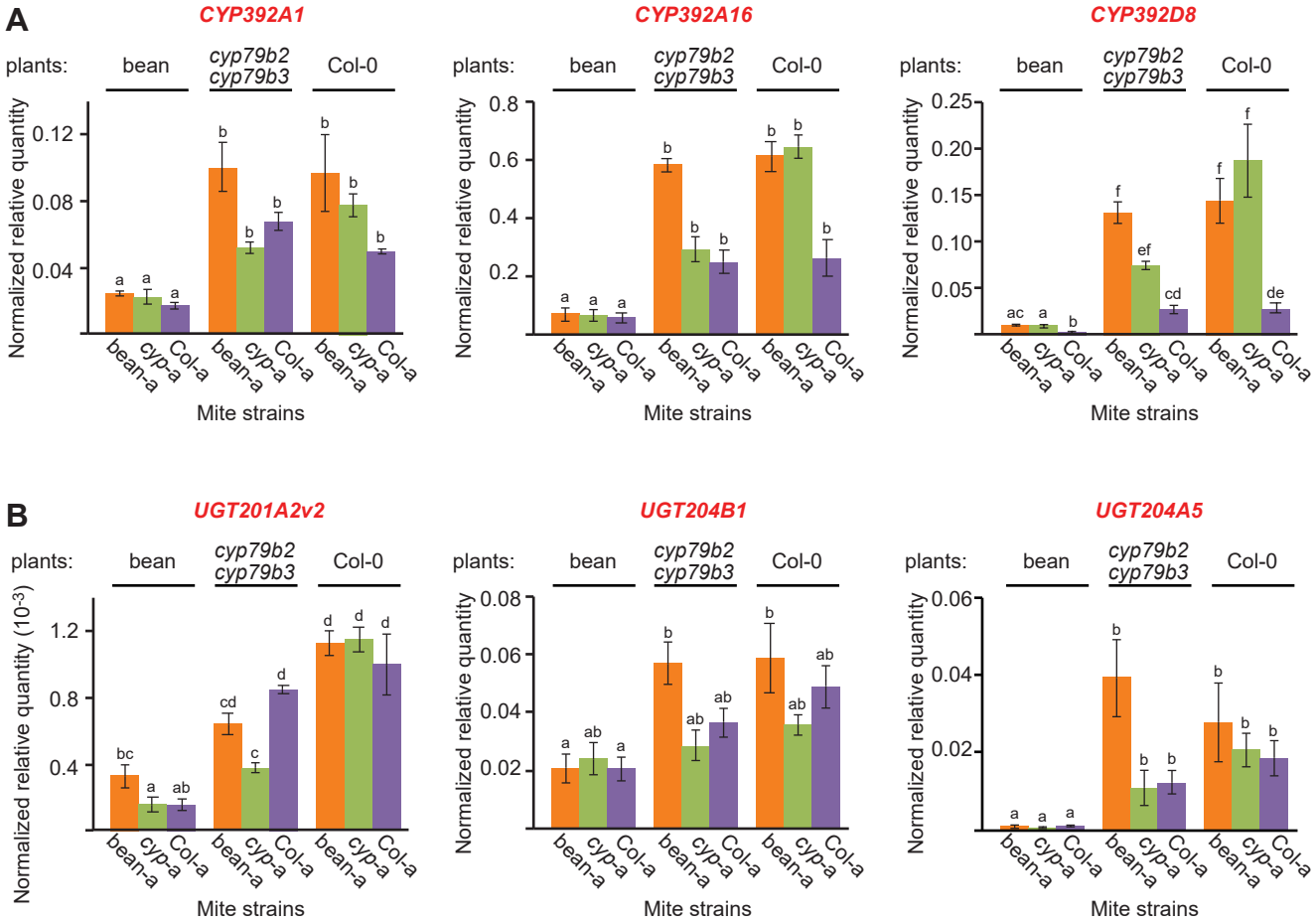

A
CYP392A1
CYP392A16
CYP392D8
plants:
bean
cyp79b2
cyp79b3
Col-0
Normalized relative quantity
Mite strains
bean-a
cyp-a
Col-a
B
UGT201A2v2
UGT204B1
UGT204A5
Normalized relative quantity (10-3)

FIGURE 5

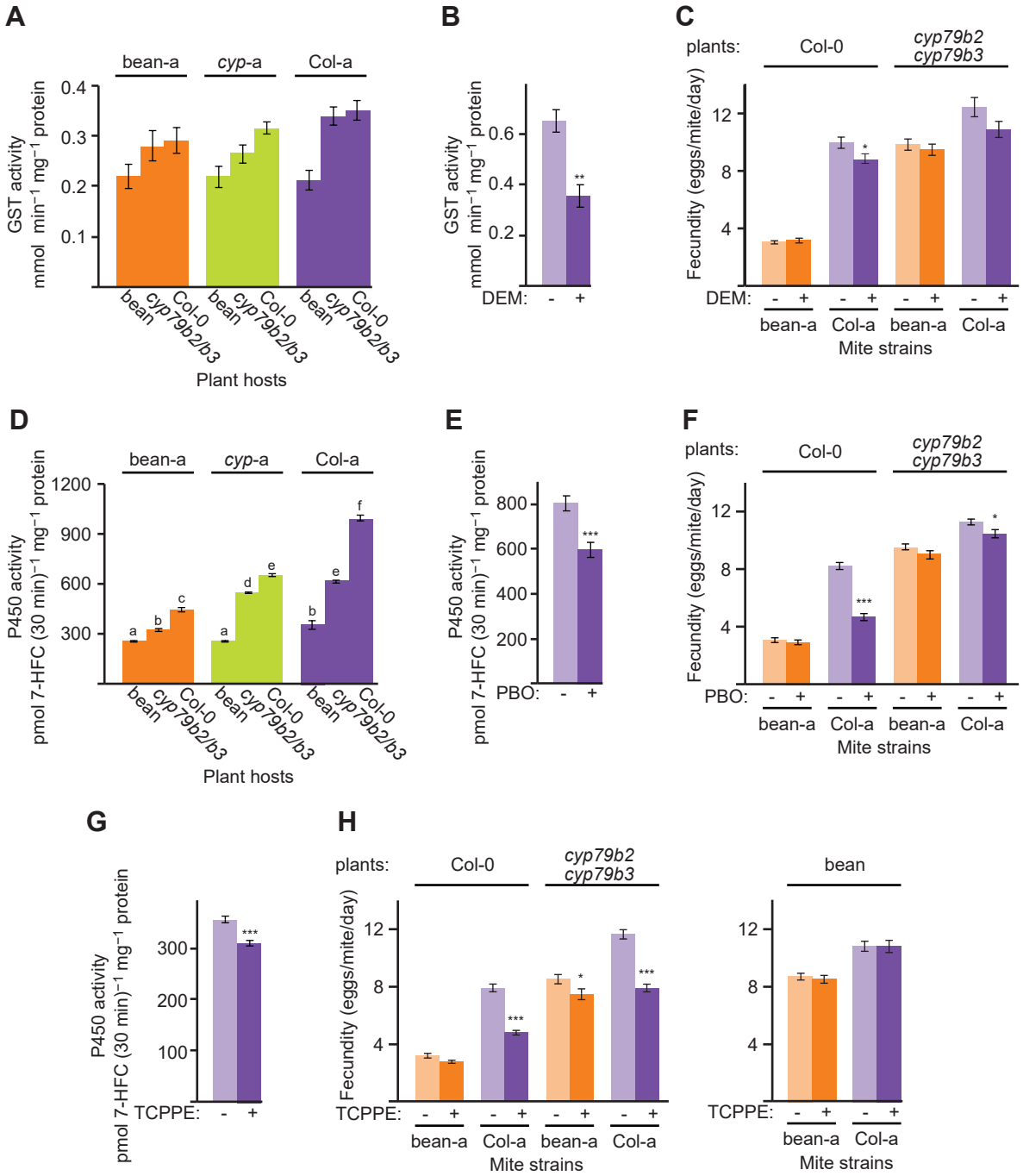
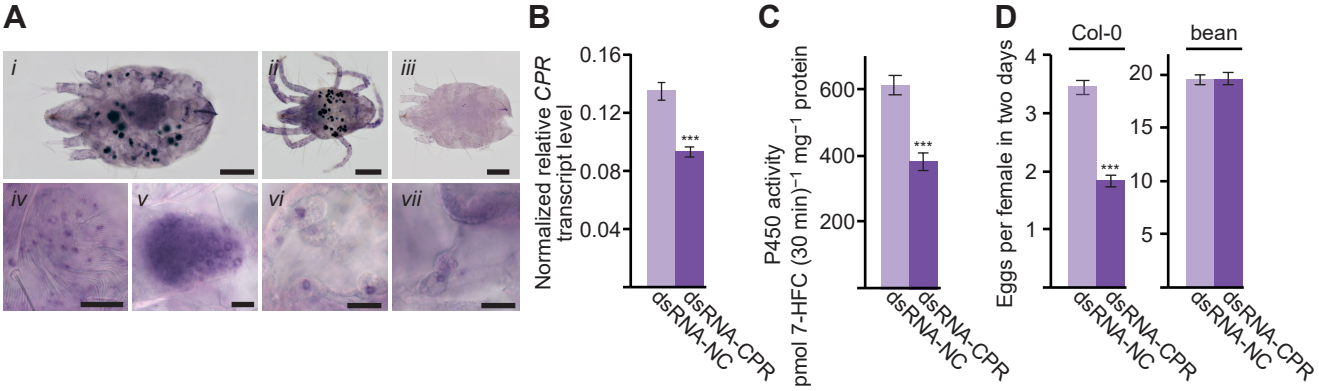

A
bean-a
cyp-a
Col-a
GST activity
mmol min−1 mg−1 protein
0.4
0.3
0.2
0.1
bean
cyp79b2/b3
Col-0
Plant hosts
B
GST activity
mmol min−1 mg−1 protein
0.6
0.4
0.2
DEM: - +
C
plants:
Col-0
cyp79b2
cyp79b3
Fecundity (eggs/mite/day)
12
8
4
DEM: - +
bean-a
Col-a
Mite strains
D
bean-a
cyp-a
Col-a
P450 activity
pmol 7-HFC (30 min)−1 mg−1 protein
1200
900
600
300
a
b
c
d
e
f
bean
cyp79b2/b3
Col-0
Plant hosts
E
P450 activity
pmol 7-HFC (30 min)−1 mg−1 protein
800
600
400
200
PBO: - +
F
plants:
Col-0
cyp79b2
cyp79b3
Fecundity (eggs/mite/day)
12
8
4
PBO: - +
bean-a
Col-a
Mite strains
G
P450 activity
pmol 7-HFC (30 min)−1 mg−1 protein
300
200
100
TCPPE: - +
H
plants:
Col-0
cyp79b2
cyp79b3
Fecundity (eggs/mite/day)
12
8
4
TCPPE: - +
bean-a
Col-a
Mite strains
bean
12
8
4
TCPPE: - +
bean-a
Col-a
Mite strains

FIGURE 6

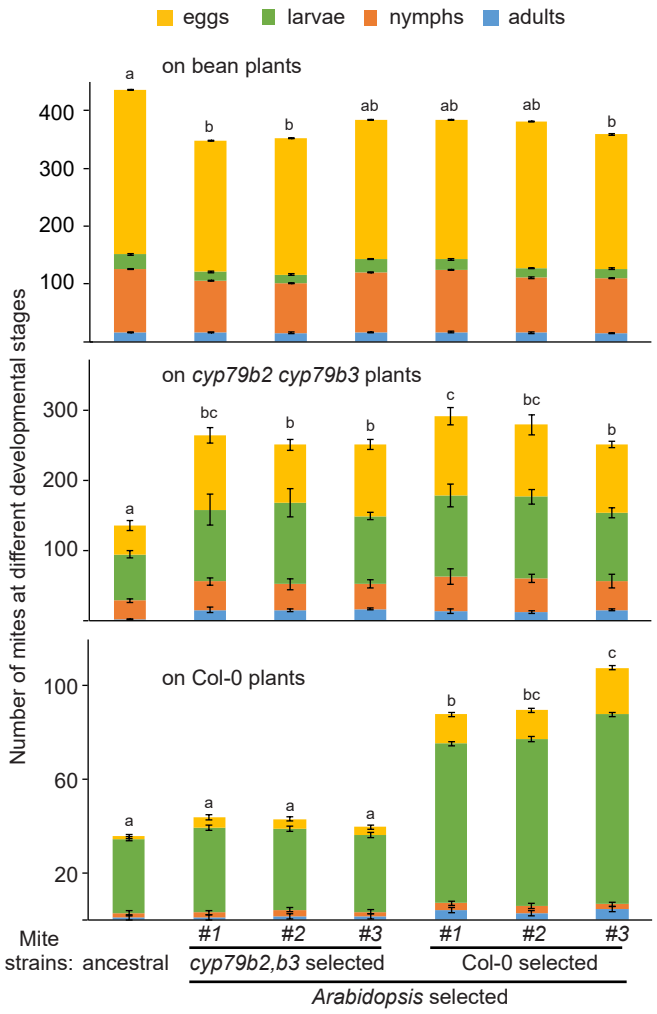

A
i
ii
iii
iv
v
vi
vii
B
Normalized relative CPR transcript level
0.16
0.12
0.08
0.04
***
dsRNA-NC
dsRNA-CPR
C
P450 activity
pmol 7-HFC (30 min)−1 mg−1 protein
600
400
200
***
dsRNA-NC
dsRNA-CPR
D
Eggs per female in two days
Col-0
bean
4
3
2
1
20
15
10
5
***
dsRNA-NC
dsRNA-CPR
dsRNA-NC
dsRNA-CPR

Supplemental Table 1. Gene-specific primer sequences used for the analysis of levels of gene expression (RT-qPCR), synthesis of dsRNA (RNAi) and preparation of probe for the *in situ* transcript localization.

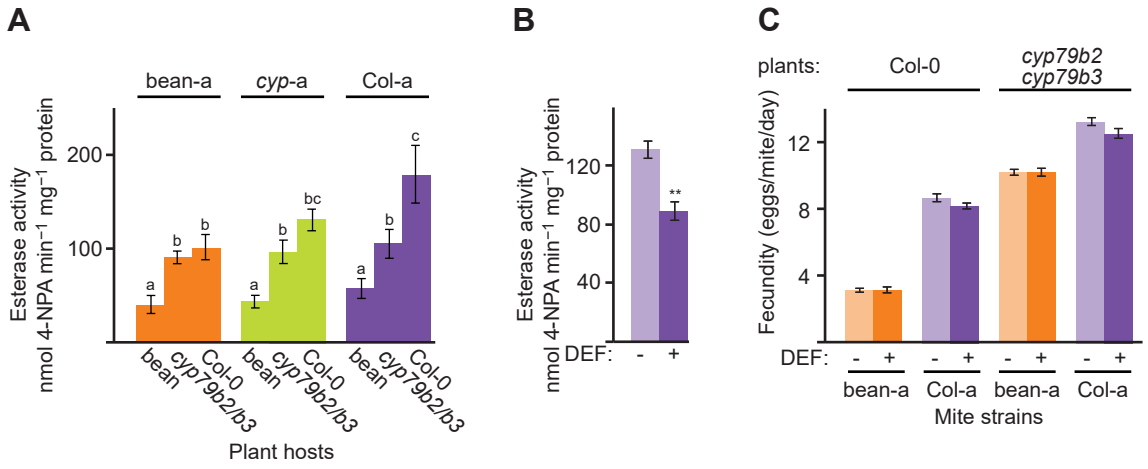
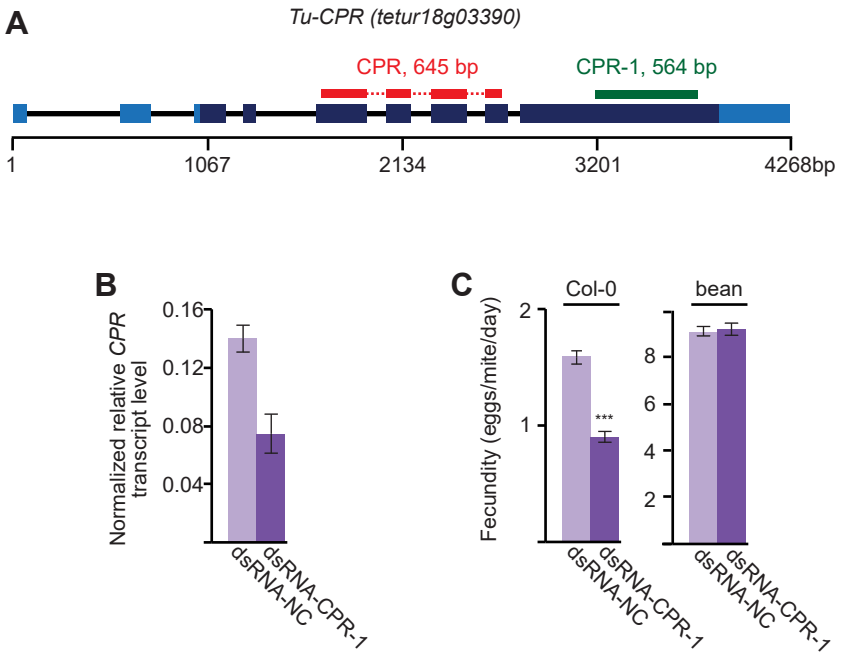
Locus ID and name	Application	Primers	Efficiency
tetur07g06410 *CYP392A1*	RT-qPCR	F: 5'-GACGCCTTCGCAAATGATGC-3' R: 5'-TAACCGTCTGTGTTCACGCC-3'	0.929
tetur06g04520 *CYP392A16*	RT-qPCR	F: 5'-TTGATTGGGCTTGCCCTCTT-3' R: 5'-AGCCAACAATCGGAAGACCC-3'	0.903
tetur03g05070 *CYP392D8*	RT-qPCR	F: 5'-ACCAGAGAGATTCCTCAGCG-3' R: 5'-AAAGCCAAAGTTGCACCAGG-3'	1.030
tetur02g02480 *UGT201A2v2*	RT-qPCR	F: 5'-TCGAGAACTGAGTGGAGTAGC-3' R: 5'-TAGCAGGCAAAGGTGTTCCA-3'	1.090
tetur02g09830 *UGT204B1*	RT-qPCR	F: 5'-GCTTCGGTTGAGAAACGTGG-3' R: 5'-AAAATCGGCATTCGCTTCGG-3'	1.000
tetur05g00090 *UGT204A5*	RT-qPCR	F: 5'-TGGACGGAAATCGTAGTGGA-3' R: 5'-AGCTCATCAAAGACCAGCGA-3'	1.090
tetur18g03590 *RP49*	RT-qPCR	F: 5'-CTTCAAGCGGCATCAGAGC-3' R: 5'-CGCATCTGACCCTTGAACTTC-3'	0.976
AT5G42650 *AOS*	RT-qPCR	F: 5'-AAATCCAACGGCGGAGAACT-3' R: 5'-TCGTCGCCAACGGTTGATAA-3'	0.984
AT4G39950 *CYP79B2*	RT-qPCR	F: 5'-GAAAAGAGGTTGTGCGGCTC-3' R: 5'-TCTCACTTCACCGTCGGGTA-3'	0.994
AT2G22330 *CYP79B3*	RT-qPCR	F: 5'-TCTACCGATGCTTACGGGATTG-3' R: 5'-TACAAGTTCCTTAATGGTTGGTTTG-3'	0.973
AT1G32640 *MYC2*	RT-qPCR	F: 5'-TCGCTTACATCAACGAGCTTAAATC-3' F: 5'- TATCTTCACTTCAATCTCCATCCCC-3'	0.900
AT5G25760 *PEROXIN4*	RT-qPCR	F: 5'-GCTCTTATCAAAGGACCTTCGG-3' R: 5'-CGAACTTGAGGAGGTTGCAAAG-3'	0.992
tetur18g03390 *Tu-CPR*	RT-qPCR	F: 5'-CCATTCTTGGCACCTATCGT-3' R: 5'-GCAAGGTGATCTCCAGCTTC-3'	0.994
tetur18g03390 *Tu-CPR*	RNAi (fragment 1)	F: 5'-[T7]-CCTCGACTTCAGCCACGTTA-3' R: 5'-[T7]-AACATCCCGAGCCATGTTCC-3'	not applicable
tetur18g03390 *Tu-CPR*	RNAi (fragment 2)	F: 5'-[T7]-ACAAACCGGTACTGCAGAGG-3' R: 5'-[T7]-TGCATACACGAACGGTCTCC-3'	not applicable
T. urticae genomic scaffold 12, position 1690614-1690995, *NC*	RNAi (negative control)	F: 5'-GCCCTCTCCTGGTTGTAAACTT-3' R: 5'-CGACCCCATCAGGCTATTGA-3'	not applicable
tetur18g03390 *Tu-CPR*	*in situ* (antisense)	F: 5'-[T7]-ACAAACCGGTACTGCAGAGG-3' R: 5'-TGCATACACGAACGGTCTCC-3'	not applicable
tetur18g03390 *Tu-CPR*	*in situ* (sense)	F: 5'-ACAAACCGGTACTGCAGAGG-3' R: 5'-[T7]-TGCATACACGAACGGTCTCC-3'	not applicable

[T7]: T7 RNA Polymerase promoter sequence, 5'-TAATACGACTCACTATAGGG-3'

SUPPLEMENTAL FIGURE 1



SUPPLEMENTAL FIGURE 2



SUPPLEMENTAL FIGURE 3