

MAPK drives UGTs metabolic resistance to neonicotinoids in whitefly by regulating the transcription factor HR39

Corresponding Author: Professor Xin Yang

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript entitled "MAPK drives the metabolic resistance of UGT to neonicotinoids by regulating the transcription factor HR39" presents a thorough and well-executed study demonstrating that uridine diphosphate (UDP)-glycosyltransferase UGT356B1 plays a key role in the detoxification of neonicotinoid insecticides in the whitefly *Bemisia tabaci*. The authors further elucidate part of the molecular pathway responsible for the upregulation of UGT356B1 in response to neonicotinoid exposure.

The study employs multiple experimental approaches to support its claims, which appear robust and reliable. Notably, the identification of several molecular actors, including the transcription factor HR39, involved in the regulation of UGT356B1 is novel, as there is limited data on the role of this TF in upregulating detoxifying enzymes.

However, the manuscript has some limitations, primarily related to stylistic inconsistencies and logical discontinuities in several sections, particularly the discussion, which reduce the clarity and readability of the text. Addressing these issues will be essential before the manuscript can be considered for publication.

1. uridine diphosphate (UDP)-glycosyltransferases are abbreviated as both UGTs and UGT. UGTs is preferable when talking about the group of enzymes (e.g., the title).
2. "in vivo", "in vitro", "cis" needs to be italicized throughout the manuscript (e.g., line 26, line 62).
3. Line 37 – substitute insecticide with pesticides.
4. Line 45 – "undermining healthcare" sounds odd. If the authors are referring to problems with vectors of human disease this needs to be better addressed. I would substitute with "undermining vector-control programs".
5. Line 47 – "elevated detoxification enzymes" Elevated expression, activity? This is missing in the sentence.
6. Line 49-52 – "the role of UGTs in resistance", not "resistance of UGTs".
7. Line 56 – substitute the word "substances" with "molecules" or similar.
8. Line 65 – First time citing *Drosophila melanogaster*. The complete name is required. Throughout the manuscript this species is addressed as *Drosophila*, but the correct form should be *D. melanogaster*.
9. Line 66 – "... in response to UP-TAKE of exogenous substances."
10. Line 67 – *N. lugens*, this species was already cited (also *B. tabaci* few lines below). I will pinpoint only this examples but there are several throughout the manuscript. Please double check.
11. Line 82 – specify in which species the mutation has been identified.
12. Line 87 – specify that this has been identified in *B. tabaci*.
13. Line 93 – no transcriptomic analysis are included in the manuscript.

RESULTS

14. Line 107 – in material and methods it is indicated that R1 and R2 were derived from S1. This needs to be addressed also here. I think it is also necessary to specify that R1 and R2 have been selected with the two compounds.
15. Line 110 – abbreviation of inhibitors needs to be indicated here.
16. Line 109-113 – rephrase, not clear and contains repetitions.
17. Line 116 – why these UGTs were selected? Or they are all the UGTs present in *Bemisia*? Furthermore no information on how these genes/transcript were identified is reported. Curated genome annotation? BLAST? Add in M&M.
18. Line 117 – specify "in all stains".
19. Line 126 – better to say "in strains resistant to both TMX and IMI"
20. Line 129 – "...encode 427AA. The resulting protein was predicted to be..."
21. Line 131-134 – this section is not clear: there are two conserved motif but then there is a reference to domains that are either conserved or variable?

22. Line 136 – specify the doses of insecticides used.
23. Line 43: plants?
24. It is not addressed why S strain does not present reduction of expression after RNAi.
25. Line 153 – maybe briefly explain what the *D. melanogaster* strain is and what is the aim of the experiment. Furthermore as it is written now it seems that the strain is overexpressed, which does not make much sense.
26. Line 192 – put a reference at the end of the sentence.
27. Imidacloprid is sometime abbreviated as IMI, other as IMD.
28. Line 205 and 207 – figure references are inverted.
29. Line 213 – knock down.
30. Line 216 – It seems that the differences in protein levels of ERK and P38 are only visible for phosphorylated forms of the proteins. Why?
31. Line 217 – briefly describe the action of activators and inhibitors. Changes in protein levels of UGT356B1 are barely visible. It would be better to use a more sensitive assay to measure
32. Line 233 – quickly cite that the TF of *Bemisia* were cloned.
33. Line 239-240 – quickly explain which essential part of HR39 are mutated in DN1 and DN2
34. Line 258 – change the title. As it is now it seems that HR39 was artificially overexpressed to study its function.

DISCUSSION

- Line 287 – I think the sentence should be changed into “... widely involved in the development of resistant in whiteflies.”
Remove “whiteflies were no exception”
Line 289 – plants?
Line 293-295 – repetition.
Line 297 – only some UGTs showed overexpression.
Line 313-317 – the sentence is not clear, rephrase.
Line 320-322 – I don’t understand the rationale behind this sentence: please argue in the manuscript on why you arrived to this conclusion.
Line 326- phase I and II are cited a bit out of the blue, although the explanation is provided just below. I would rephrase to first define what phase I and II are and then explain the rest. Still, what does it mean that your study suggest that UGT356B1 may not solely rely on direct catalytic ability? Why this conclusion? And the sentence below seems to be in contrast with this statement.
Line 343 – this has already been reported above.
Line 345 – there is an hanging “t”.
Line 349 – the example provided are not on neonicotinoids but on Bt toxins, so I would not start the sentence with “for example” as this are not really examples of the sentence above.
Line 366 – domains abbreviation were already defined.
Line 374 – another work on mites demonstrated that HR96 can regulate several detoxification enzymes:
<https://www.nature.com/articles/s41467-023-40778-w>
Line 380 – the concluding paragraph could be strengthened to more clearly summarize the main findings and their implications.

MATERIALS AND METHODS

35. Line 389 – names TMX-resistant and IMI resistant are confusing. I guess this is due to selection of the strains on those pesticides, however based on toxicity results there is cross-resistance. Furthermore it is necessary to indicate how many generation of selection occurred and the concentration of insecticides used for selection.
36. Line 408 – “total protein” indicate how protein extraction was executed.
37. Line 412 – “... second round of washing (5 times)” not clear. Was it washed five times?
38. Line 435 – “as well as”
39. Line 475 – indicate PCR conditions or refer to Supp. tables.
40. Line 548 – what does “conserved mutant forms” means?

GENERAL COMMENTS

The authors should discuss and highlight why all the strains investigated (both field strains and artificially selected) seems to have evolved the same resistance mechanism.
I suggest to revise the entire manuscript for stylistic inconsistencies that might have not been indicated in my comments above.

I suggest to include in the results section a brief explanation of experimental procedure and rationale of experiments to improve readability. Above there are some specific comments addressing this but it should be applied more broadly.

FIGURES and figure caption.

Figure 1

A-B: IMI and TMX abbreviations are not used in the figure but are reported in figure caption. There is no color legend for dose-response curves.

C: is too small. Can become supp. figure.

Figure2

A-B: Indicate dose of TMX in the figure or figure legend.

C: field strains: I think it is not correct to refer to R1 and R2 as field strains (as commented above).

Description of panel E and D is inverted.

Figure 4

Define abbreviations of Total-ERK (T-ERK) and phosphorylated ERK. The same for p38.

Line 859 – which lane is activator and which one is inhibitor.

Figure 5

Line 878 – define what activity.

Mut1 sequence provided in panel F is wrong (=WT).

Figure 6D – I find this panel unclear. I would be consistent with the other panel/figures and use bar chart. Also the title “mortality of RNAi” is incorrect. Mortality of *B. tabaci* after RNAi treatment.

GRAPHICAL ABSTRACT

I think the “wi-fi” symbol should be inverted to show that MAPK cascades acts on ERK and p38. As it is now it seems to be the opposite.

Reviewer #2

(Remarks to the Author)

The manuscript by Du et al investigated the source of metabolic resistance in field- collected population of *Bemisia tabaci*. The authors screened select Uridine diphosphate (UDP)-glycosyltransferases (UGTs) genes and identified increased in expression and biochemical activity of BtUGT356B1. Further investigation through gene knockdown via RNAi and qPCR indicated a collection between BtUGT356B1 expression and resistance to thiamethoxam and imidacloprid. Furthermore, in vivo and in vitro studies MAPKs P38 and ERK identified via the TF HR39 as the regulatory mechanism for BtUGT356B1. The manuscript provides evidence of the role of metabolic resistance and potential regulatory mechanism. Before the manuscript is worthy of being considered for publication in this journal but the authors need to address the issues list below before been accepted.

1. The current structure of the manuscript indicates a fishing expedition approach on the link between BtUGT356B1 and resistance in *B. tabaci*. The authors made no attempt to elucidate the potential contribution of other source of resistance (P450s, GTS, esterase, ABC transporters and SNPs). Not addressing these other pathways to insecticide resistance can overstate the contribution of BtUGT356B1 to resistance.
2. What about inductive and constitutive expression of BtUGT356B1 under exposure to THI and IMI.
3. It is surprising that the authors conducted no genome scan or transcriptomic analysis to have a comprehensive understanding of the mechanism of the genetic basis of resistance in the *Bemisia* populations.
4. What is the rationale for selecting the candidate UGT genes that were screened to identify BtUGT356B1.
5. The abstract and introduction can be improved with better transition and connection between pesticide usage and resistance and the need to connect regulatory mechanism to source of resistance.
6. Line 94: can the authors qualify the strength of UGT's contribution to resistance in this study.
7. How sure are the authors that UGT is the sole source of resistance (monogenic vs polygenic)
8. Provide the R2 values for figures 2A and 2B.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors put substantial effort in addressing the comments. However, although the clarity of the manuscript improved, several aspects need further clarification.

- “Regulatory mechanisms, as a bridge linking pesticide use and metabolic resistance phenotypes, are playing an increasingly prominent role.” This sentence is not clear.
- Line 71: “and promoting” is in italics.
- Titles in the Results section are inconsistent: some summarize what was done, while others highlight the main findings. They should be standardized for clarity.
- Line 121: “10 candidate UGT” specify here that this is based on a previous study.
- Lines 142–145: A major concern is how 50 mg/L would affect the S strain before RNA extraction, as this concentration is considerably above the LC50 for the S strain (i.e., not sublethal). I understand that LC50s were measured after 24 h, while expression was measured earlier; however, mortality would still be expected in S (or at least the insects should be severely affected). Was mortality observed in the S strain? Were RNA extractions performed only from surviving individuals? Furthermore, these data indicate not only that basal BtUGT356B1 expression is higher in R strains, but also that R individuals can induce further expression when exposed to the pesticides. This distinction should be clearly stated in the Results. The lack of inducible expression in the S strain is interesting however it is probably due to the high dosage (e.g., individuals were severely affected and the endogenous response was inhibited)?
- Lines 145–146: In Figure 2B, UGT356B1 protein levels at time point 0 are very similar in S and R strains. The actin loading control suggests that this is not due to differences in total protein loaded. Why is this the case? This seems to contrast with Figure 2F, where UGT356B1 in S appears clearly lower than in R strains.
- Section “MAPK Signaling Pathway Regulates BtUGT356B1 Transcription”: I understand that phosphorylated ERK represents the active form of the protein. What may be confusing is that RNAi targeting ERK appears to affect only the phosphorylated (active) form, while total ERK protein levels remain unchanged. This outcome is biologically plausible, as ERK kinase activity and phosphorylation are not strictly dependent on total ERK abundance in a highly dynamic signaling system. RNAi preferentially impact newly synthesized, signaling-competent ERK, thereby reducing the active fraction without measurably altering total protein levels if they are high. This distinction should be explicitly addressed in the Discussion (now I believe it is not clear enough), and the discrepancy between total ERK and phosphorylated ERK should also be clearly described in the Results section (not indicated in the current version). Furthermore expression data of ERK would be a welcome addition as they would prove the effective downregulation of the gene. This was done for both RNAi

experiment targeting UGT356B1 and HR39. This section should be rewritten including a more detailed analysis of the results. Furthermore, in Figure 4D, the S strain shows phosphorylated ERK comparable to resistant strains, while total ERK protein is considerably lower. This appears to contradict the explanation that phosphorylated ERK reflects ERK activity. Is this possible? Since phosphorylated ERK is a subset of total ERK, how can it be higher than total ERK? These observations should be discussed and clarified; otherwise, the protein data appear confusing.

- Line 245: "was" seems out of place

- Line 312: UGT2? Is there something missing?

- Line 321-329: I would integrate what you described in your response about the low baseline expression. Mortality was already very high and does not provide strong evidence.

- Line 332-336: This part remains unclear. From your response, it appears that you assume that if resistance relied mainly on direct catalysis, TMX and IMI should show much stronger binding than other substrates. However, detox enzymes are generally generalists and can metabolize several substrates. Catalytic activity is not solely determined by binding affinity. What can safely be stated is that BtUGT356B1 can bind TMX and IMI, and no obvious structural specialization is apparent. Furthermore, the sentence "It may also indirectly enhance overall resistance through synergistic effects or by regulating other genes to influence cellular detoxification pathways" seems disconnected from the docking analysis and is not entirely correct. What does it mean that UGT356B1 can regulate other genes? I suggest removing this sentence. Possible synergism with other detoxification enzymes, such as P450s or CCEs, is already discussed elsewhere.

- Line 358-360: "but" should be removed. "especially the highly expressed individual subtypes..." your data only validate a single UGT. I would stick to that without adding speculations about other UGTs.

- Line 376: "stimulating conditions". What does it mean? No stimulus has been described. These data are about RNAi experiment on a resistant strain.

- Line 375-385: I find this clarification not satisfactory. Please try to modify as described in a comment above. This section should explain why after RNAi only phosphorylated proteins are affected. How that can influence expression of UGTs.

- Line 413: "exhibit a unified resistance mechanism" this claim can not be proved as only the role of UGTs has been investigated. Different strains, which also present different resistant level, might have evolved other mechanisms (as also described above in discussion). Needs to be rephrased.

- References to supplementary figure 1c, 2, and 3 are totally absent in the text.

Please, double check for typos, words in italics and be sure all supplementary material is cited in the appropriate way.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)

As an additional reviewer, I primarily focused on concerns previously raised by Reviewer 1 and whether these issues have been satisfactorily addressed in the revised manuscript. Overall, the manuscript has been substantially improved through the revision process, and most of the concerns raised by Reviewer 1 have been adequately addressed. The revisions have strengthened both the presentation and interpretation of the data. Nevertheless, a few points may still benefit from further clarification to improve the rigor of the study and the clarity of conclusions.

Regarding Reviewer 1's comment #1, the revised text improved but remains somewhat general. The discussion could benefit from a more mechanistic description of how transcriptional regulation contributes to metabolic resistance. For example: "During the evolution of metabolic resistance, transcriptional regulatory mechanisms play a critical role in connecting insecticide exposure with resistance phenotypes. Insecticide-induced or constitutively activated transcription factors can upregulate detoxification genes, including cytochrome P450s, glutathione S-transferases, and carboxylesterases, thereby enhancing insecticide metabolism and detoxification. Increasing evidence suggests that such regulatory mechanisms are important contributors to the evolution of metabolic resistance in insect pests."

Regarding Reviewer 1's comment #5, the authors have adequately clarified the timing of gene expression measurements relative to LC50 determination, and the distinction between constitutive and inducible expression has been appropriately incorporated into the results section. However, some additional details would help readers to better evaluate the data. The author states that "only those individuals that remained alive and in good physiological condition after insecticide treatment were selected for RNA extraction". It would therefore be useful to indicate how many individuals were excluded prior to RNA extraction, and whether all surviving insects were pooled or only a selected subset? Such information would help readers assess the potential impact of survivor bias. Given that 50mg/L is close to the LC50 of the S strain, selecting only the healthiest surviving individuals could potentially influence the observed expression patterns.

In addition, the newly added discussion suggesting that "high-dose insecticides may directly damage physiological functions... inhibit stress response pathways..." is reasonable and plausible. However, as direct evidence for this is not provided in this study or by references, it maybe more appropriate to present this interpretation as a possible explanation rather than a definitive conclusion (e.g. "one possible explanation is that....").

A related point is that the alternative explanation raised by Reviewer 1 has not been directly explored experimentally. Specially, the lack of inducible expression in the S strain may reflect the treatment conditions rather than an intrinsic inability to induce expression. Because the same concentration (50mg/L) was applied to both S and R strains despite their substantially different LC50 values, the physiological stress experienced by the two strains is unlikely to be equivalent,

therefore, the possibilities that treatment conditions contributed to the observed differences should be acknowledged as a limitation in the discussion.

Regarding Reviewer 1's Comment #6, the authors have provided additional clarification regarding the apparent discrepancy between Fig 2B and 2F, however, some uncertainty remains. The explanation that the similarity observed in Fig 2B may reflect either the comparable basal expression levels or experimental error does not completely reconcile the difference between the two figures. If these data were generated from independent experimental batches, it would be helpful to provide additional information regarding normalization procedures, quantitative analysis, and any limitations associated with cross-batch comparisons.

Finally, the statement "RNAi preferentially targets newly synthesized, signaling-competent ERK" appears central to the author's interpretation of the ERK phosphorylation data. If available, it would be helpful for the authors to provide experimental evidence or appropriate literature reference supporting this mechanism.

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Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The manuscript entitled "MAPK drives the metabolic resistance of UGT to neonicotinoids by regulating the transcription factor HR39" presents a thorough and well-executed study demonstrating that uridine diphosphate (UDP)-glycosyltransferase UGT356B1 plays a key role in the detoxification of neonicotinoid insecticides in the whitefly Bemisia tabaci. The authors further elucidate part of the molecular pathway responsible for the upregulation of UGT356B1 in response to neonicotinoid exposure.

The study employs multiple experimental approaches to support its claims, which appear robust and reliable. Notably, the identification of several molecular actors, including the transcription factor HR39, involved in the regulation of UGT356B1 is novel, as there is limited data on the role of this TF in upregulating detoxifying enzymes.

However, the manuscript has some limitations, primarily related to stylistic inconsistencies and logical discontinuities in several sections, particularly the discussion, which reduce the clarity and readability of the text. Addressing these issues will be essential before the manuscript can be considered for publication.

RESPONSE: We sincerely appreciate your meticulous review and invaluable guidance on our submitted manuscript. Your professional knowledge and experience have played an important role in promoting my research. We have carefully studied your comments and revised the paper accordingly. Here are the details of the changes and responses:

1. uridine diphosphate (UDP)-glycosyltransferases are abbreviated as both UGTs and UGT. UGTs is preferable when talking about the group of enzymes (e.g., the title).

RESPONSE: We sincerely appreciate the reviewer's meticulous attention to the proper usage of the term "UDP-glycosyltransferase" (UGT).

The title has been revised to: "MAPK drives the metabolic resistance of UGTs to neonicotinoids by regulating the transcription factor HR39." We have thoroughly reviewed the manuscript to ensure that the usage of "UGTs" (for the family) and "UGT" (for specific enzymes/subtypes) fully complies with the above guidelines and maintains terminological consistency throughout the manuscript.

2. "in vivo", "in vitro", "cis" needs to be italicized throughout the manuscript (e.g., line 26, line 62).

RESPONSE: We sincerely appreciate the reviewer's meticulous attention to the formatting details. We have implemented the following revisions: all instances

of “in vivo”, “in vitro”, and “cis” have been italicized in the new version.

3. Line 37 – *substitute insecticide with pesticides.*

RESPONSE: We appreciate the reviewer's suggestion and have revised the manuscript to replace "insecticide" with "pesticide". Please see Line 38 in the new version.

4. Line 45 – *“undermining healthcare” sounds odd. If the authors are referring to problems with vectors of human disease this needs to be better addressed. I would substitute with “undermining vector-control programs”.*

RESPONSE: We greatly appreciate the reviewer's insightful suggestion to clarify the terminology. We agree that "undermining healthcare" may be ambiguous. As suggested, we have substituted it with "undermining vector-control programs". Please see Line 46-47 in the new version.

5. Line 47 – *“elevated detoxification enzymes” Elevated expression, activity? This is missing in the sentence.*

RESPONSE: We sincerely thank the reviewer for this critical observation. The original phrasing (“elevated detoxification enzymes”) was indeed imprecise, as it did not distinguish between enzyme expression and activity. We modified it to an “increase in detoxification enzyme activity”. Please see Line 49 in the new version.

6. Line 49-52 – *“the role of UGTs in resistance”, not “resistance of UGTs”.*

RESPONSE: We appreciate the valuable comments provided by the reviewers. We have made the wording changes in the revised draft. Please refer to line 51 of the new version.

7. Line 56 – *substitute the word “substances” with “molecules” or similar.*

RESPONSE: We appreciate the reviewer's suggestion to enhance terminological precision. The “substances” has been replaced with “molecules”, Please see Line 57 in the new version.

8. Line 65 – *First time citing Drosophila melanogaster. The complete name is required. Throughout the manuscript this species is addressed as Drosophila, but the correct form should be D. melanogaster.*

RESPONSE: We sincerely appreciate the reviewer's meticulous attention to taxonomic nomenclature. We fully agree that consistent use of scientific names is essential for academic rigor. The first instance (Introduction, line 68) now reads: "In *Drosophila melanogaster*, ..." All following mentions have been standardized to "*D. melanogaster*" (e.g., Results line 162, Discussion 398,

Methods line 508).

9. Line 66 – “... in response to UP-TAKE of exogenous substances.”

RESPONSE: We thank the reviewer for the rigorous guidance on terminology. Please see Line 69 in the new version.

10. Line 67 – *N. lugens*, this species was already cited (also *B. tabaci* few lines below). I will pinpoint only this examples but there are several throughout the manuscript. Please double check.

RESPONSE: We sincerely appreciate the reviewer's meticulous and rigorous work. We have meticulously revised such oversights throughout the manuscript.

11. Line 82 – specify in which species the mutation has been identified.

RESPONSE: We appreciate the reviewer's insightful comment. We have pointed out that this mutation is a key site related to neonicotinoid resistance found in whitefly. Please see Line 83 in the new version.

12. Line 87 – specify that this has been identified in *B. tabaci*.

RESPONSE: We sincerely appreciate the reviewer's comment. We have carefully revised the manuscript as follows: Recent findings suggest that *CYP6CM1* is under regulation of CREB through the MAPK pathway, leading to its overexpression and promotion of IMI resistance in *B. tabaci*.

13. Line 93 – no transcriptomic analysis are included in the manuscript.

RESPONSE: We sincerely appreciate the reviewer for the meticulous comments regarding the transcriptomic analysis. In response to this valuable feedback, we have clarified in the revised manuscript that the previously mentioned transcriptomic data was only intended to provide the background for UGT gene selection and was not included in the current study. To address this limitation, we have added a new paragraph in the Discussion (line 361-365) detailing the subsequent transcriptomic research plan, which aims to systematically investigate the impact of neonicotinoid insecticides on UGT gene transcriptional regulation. We sincerely apologize for any potential misunderstanding caused by the original wording and hope that these revisions adequately address the reviewer's concerns.

The revised sentence is now (line 94-98): “To systematically investigate the direct metabolic capacity of UGTs for neonicotinoid insecticides and elucidate their potential upstream transcriptional regulatory mechanisms, we employed classic molecular biology techniques as the core research approach, utilizing multi-method validation to reveal their functional characteristics.”

RESULTS

14. Line 107 – in material and methods it is indicated that R1 and R2 were derived from S1. This needs to be addressed also here. I think it is also necessary to specify that R1 and R2 have been selected with the two compounds.

RESPONSE: We thank the reviewer for these constructive comments. We fully agree that these details are crucial for understanding our experimental design and have revised this section to: "The sensitivity of TMX and IMI was tested in laboratory strains (S^{#1}, R^{#1} and R^{#2}) and field-collected strains (FR^{#1}, FR^{#2}, FR^{#3}, FR^{#4} and FR^{#5}) from different regions of China using bioassay."

Meanwhile, the Materials and Methods section (Lines 441-449) has been revised as follows: "The *B. tabaci* MED strain was initially collected from tomato plants in Hangzhou, Zhejiang (30°20'N, 120°38'E) in 2011. Using the two-way method, this strain was divided into three batches with identical genetic backgrounds. Two batches were then used to construct laboratory-resistant strains (R^{#1} and R^{#2}) by selection with thiamethoxam and imidacloprid at a LC₅₀ concentration (100 mg/L), respectively. The remaining batch was used for diagnostic dose bioassays on the offspring of single-pair, non-intercrossed whiteflies to establish a laboratory-susceptible strain (S^{#1}). This susceptible strain has been maintained for over a decade without exposure to any insecticides."

15. Line 110 – abbreviation of inhibitors needs to be indicated here.

RESPONSE: We sincerely appreciate your careful review. Regarding the abbreviation of inhibitors, we have now added the full terms with their corresponding abbreviations upon first mention in the section. Specifically: "we treated all strains with UGT inhibitors (5-nitrouracil (5-Nul) and sulfinpyrazone (Sul))," Please see Line 113-115 in the new version.

16. Line 109-113 – rephrase, not clear and contains repetitions.

RESPONSE: We sincerely thank you for careful review and have rephrased it as: "To investigate the role of UGT enzymes in TMX and IMI resistance, we exposed all strains to UGT inhibitors (5-nitrouracil (5-Nul) and sulfinpyrazone (Sul)). As shown in Supplementary Table 3, treatment with these inhibitors significantly reduced the survival rate of resistant whitefly. The mortality response curves for resistant strains demonstrated a dose-dependent decline. In contrast, the sensitive strain showed no significant change in mortality (Fig.1A and 1B)." Please see Line 113-119 in the new version.

17. Line 116 – why these UGTs were selected? Or they are all the UGTs present in Bemisia? Furthermore no information on how these genes/transcript were identified is reported. Curated genome annotation? BLAST? Add in M&M.

RESPONSE: We sincerely appreciate your insightful questions regarding the

selection rationale and identification methods of UGT genes in our study. The fundamental principles for screening candidate UGT genes involved analyzing the differential expression and functional characteristics of UGT family members, combining bioinformatics predictions with experimental validation to ultimately identify the target genes.

Firstly, based on the analysis of our laboratory's preliminary transcriptome data (Accession No.: GSE42102) (Ref.1), these genes exhibited significantly higher expression levels in resistant strains compared to susceptible strains. Secondly, bioinformatics analysis revealed that these genes share homology with known insect UGTs involved in pesticide metabolism. Finally, bioinformatics results indicated that in the whitefly genome, these genes were located in regions previously identified as detoxification enzyme gene clusters.

For the identification of candidate UGTs, we relied on previously published transcriptome data, which is publicly available in the NCBI database, to identify and clone the candidate UGTs. The genomic structure and BLAST analysis of UGT genes were sourced from NCBI. Specifically, we obtained the full-length cDNA sequences from the transcriptome data, designed primers for qPCR validation, and constructed phylogenetic trees for functional clustering.

As described in the supplementary materials (Supplementary Figure 2), comprehensive records were made for each UGT gene, including their genomic locations, transcript lengths, domain structures, and their phylogenetic relationships within the genome. We have incorporated this information into the Materials and Methods section.

References

- [1] Yang, N. et al. Transcriptomic and proteomic responses of sweet potato whitefly, *Bemisia tabaci*, to thiamethoxam. *PloS one*, **8**, e61820(2013).

18. Line 117 – specify “in all stains”.

RESPONSE: We thank the reviewer for the meticulous examination and have made the corresponding revisions, please see Line 122 in the new version.

19. Line 126 – better to say “in strains resistant to both TMX and IMI”

RESPONSE: We thank the reviewer for the meticulous examination and have made the corresponding revisions, please see Line 131-132 in the new version.

20. Line 129 – “...encode 427AA. The resulting protein was predicted to be...”

RESPONSE: We thank the reviewer for the detailed revision suggestions. The manuscript has been revised accordingly. please see Line 134 in the new version.

21. Line 131-134 – this section is not clear: there are two conserved motif but then there is a reference to domains that are either conserved or variable?

RESPONSE: We thank the reviewer for identifying the unclear expression. We have rephrased it as follows: “The AA sequence contains two sugar donor-binding regions (DBR1 and DBR2) and a UGT signature motif ([FAV]-[LIVMF]-[TS]-[HQ]-[SGAC]-G-x(2)-[STG]-x(2)-[DE]-x(6)-P-[LIVMFA]-[LIVMFF]-x(2)-P-[LMVFIQ]-x(2)-[DE]-Q, where x denotes any amino acid) , which are respectively located in the central region of the C-terminal domain (Supplementary Fig. 1B).” Please see Line 136-141 in the new version.

22. Line 136 – specify the doses of insecticides used.

RESPONSE: We appreciate the reviewer's request for highlighting this important detail. Upon review, we have updated the manuscript to explicitly specify the doses: “Expression of *BtUGT356B1* in adult R^{#1} increased 1.8- and 2.4-fold after being exposed to TMX (50 mg/L) for 6 hours and 12 hours, respectively. R^{#2} strain was exposed to IMI (50 mg/L) for 12 hours resulted in a 1.6-fold increase (Fig. 2A).” Please see Line 143-144 in the new version.

23. Line 43: plants?

RESPONSE: We apologize for the spelling mistake here. The correct term should be “strains”, which has been amended in the revised version.

24. It is not addressed why S strain does not present reduction of expression after RNAi.

RESPONSE: We sincerely appreciate the meticulous review of the RNAi experimental results for the target gene in our manuscript. In response to your insightful observation regarding the absence of significant target gene expression reduction in the susceptible strain, we have conducted a comprehensive analysis based on our experimental data, focusing on two key aspects:

1. Differential RNAi silencing efficiency between strains

The observed variation in RNAi-mediated silencing efficiency may stem from the substantially lower baseline expression levels of both target mRNA and protein in the susceptible strain compared to the resistant strain (as demonstrated by WB). This inherent low expression level in the susceptible strain renders the relative reduction post-RNAi treatment less pronounced. Moreover, minor expression changes at this low expression level are more susceptible to technical variability, potentially below the detection threshold of qPCR assays.

2. Functional inhibition despite non-significant mRNA changes

Although the mRNA levels of the target gene in the susceptible strain did not exhibit statistically significant changes post-RNAi treatment, our functional validation experiments on *B. tabaci* resistance phenotypes revealed an increased mortality rate in the susceptible strain upon target gene silencing. This observation suggests that the target gene function was still partially

inhibited, despite the absence of significant mRNA level reduction.

We have revised the manuscript accordingly, incorporating a detailed discussion about the non-significant expression changes in the susceptible strain (line 321-329). We are truly grateful for your valuable suggestions, which have significantly enhanced the rigor and clarity of our study.

25. Line 153 – maybe briefly explain what the D. melanogaster strain is and what is the aim of the experiment. Furthermore as it is written now it seems that the strain is overexpressed, which does not make much sense.

RESPONSE: We sincerely appreciate your insightful question regarding our experimental approach. The *D. melanogaster* strain used in the experiment was the wild-type (w (1118)), which had been genetically modified to carry the attP40 docking site on chromosome 2 (pre-inserted via the PhiC31 integrase system) for subsequent site-specific integration of exogenous genes. Additionally, we utilized Tub-GAL4 driver *D. melanogaster* (w (1118) background) and UAS-*BtUGT356B1* transgenic *D. melanogaster*. Crossbreeding was employed to achieve tissue-specific expression. This approach combines molecular cloning, site-specific integration, and Drosophila genetics to establish a heritable Drosophila line that specifically expresses *BtUGT356B1*.

The core objective of this study is to analyze the function of the *BtUGT356B1*. By comparing the mortality between overexpression strain and the wild-type strain following insecticide treatment, we aimed to obtain direct evidence of *BtUGT356B1*'s role in insecticide resistance.

Regarding your critical point that "the target gene strain appears to be overexpressed, which doesn't make much sense," we fully understand your concern. While you noted that the expression levels might exceed physiological ranges, this design has a strong theoretical basis. First, in the evolution of insect resistance, UGT family genes often achieve significant expression increases through gene amplification (Refs.1-3), and our overexpression level is intended to simulate such molecular adaptations under natural selection pressures. Second, moderately increasing target gene expression helps overcome the interference of metabolic compensation effects, thereby clearly characterizing the independent function of the specific UGT gene. Furthermore, the transgenic Drosophila strain construction method used in this study has been systematically validated for reliability in previous research (Refs. 4-6). Importantly, if transgenic *D. melanogaster* strain that overexpress *BtUGT356B1* exhibit significantly enhanced resistance, it would provide strong evidence that the gene is a sufficient factor in resistance development.

In summary, the overexpression strategy in this experimental design was implemented to unambiguously determine gene function, rather than to precisely mimic natural expression conditions. This approach is a deliberate and effective experimental design to directly test our scientific hypothesis. We sincerely appreciate your rigorous review, which has significantly contributed to

the clarity of our study.

References

- [1] Wang, L. et al. Overexpression of Multiple UDP-Glycosyltransferase Genes Involved in Sulfoxaflor Resistance in *Aphis gossypii* Glover. *J Agric Food Chem.* **69**, 5198-5205 (2021).
- [2] Wang, M.Y. et al. Functional analysis of *UGT201D3* associated with abamectin resistance in *Tetranychus cinnabarinus* (Boisduval). *Insect Sci.* **27**, 276-291 (2020).
- [3] Wen, X. et al. UGT201H1 overexpression confers cyflumetofen resistance in *Tetranychus cinnabarinus* (Boisduval). *Pest Manag Sci.* **80**, 4675-4685 (2024).
- [4] Gong, C. et al. A horizontally transferred plant fatty acid desaturase gene steers whitefly reproduction. *Adv Sci.* **11**, e2306653(2024).
- [5] Yang, X. et al. Epitranscriptomic regulation of insecticide resistance. *Sci Adv.* **7**, eabe5903 (2021).
- [6] Hu, J. et al. CYP4CS5-mediated thiamethoxam and clothianidin resistance is accompanied by fitness cost in the whitefly *Bemisia tabaci*. *Pest Manag Sci.* **80**, 910-921(2024).

26. Line 192 – put a reference at the end of the sentence.

RESPONSE: We sincerely appreciate the valuable suggestions provided by the reviewers. In response to your comments, we have added relevant citations [13] at this location to support the discussion, as detailed in the revised version (line 203).

References

- [13] Dimunová, D., Matoušková, P., Podlipná, R., Boušová, I. & Skálová, L. The role of UDP-glycosyltransferases in xenobiotic resistance. *Drug Metab Rev.* **54**, 282-298 (2022).

27. Imidacloprid is sometime abbreviated as IMI, other as IMD.

RESPONSE: We sincerely appreciate the valuable suggestion. In the revised manuscript (line 214 and line 957), we have uniformly standardized the abbreviation of "imidacloprid" to "IMI" throughout the text to comply with the field's standard conventions.

28. Line 205 and 207 – figure references are inverted.

RESPONSE: We sincerely appreciate the meticulous and detailed review. In response to your valuable comments, we have corrected the oversight at this location. Please see lines 214-218 of the revised version.

29. Line 213 – knock down.

RESPONSE: We sincerely appreciate your meticulous review. The "knocked out" has been uniformly revised to " knock down " as suggested.

30. Line 216 – It seems that the differences in protein levels of ERK and P38 are only visible for phosphorylated forms of the proteins. Why?

RESPONSE: We sincerely appreciate the reviewer for raising this highly insightful question. ERK and P38, as members of the MAPK family, rely on phosphorylation for their functional activity. The phosphorylated forms of ERK/P38 directly participate in downstream signaling, while the non-phosphorylated forms are typically inactive precursor proteins. Therefore, differences in phosphorylated protein levels better reflect the dynamic regulation of signaling pathways rather than changes in total protein quantity. Total protein levels primarily reflect gene expression and protein stability, whereas phosphorylation status indicates the degree of transient pathway activation.

Our data show that under stimulation conditions, the total protein levels of ERK and P38 remain stable, but their phosphorylation levels change significantly. This aligns with the "signal amplifier" characteristic of the MAPK pathway, where rapid signal transduction is achieved through post-translational modifications rather than protein synthesis. Therefore, our experiments demonstrate that differences in ERK/P38 protein levels are only visible in their phosphorylated forms, precisely confirming that phosphorylation is the key mechanism for rapid MAPK pathway response, while the stability of total protein levels reflects the cell's fine-tuned regulation of basal signaling. This finding provides important insights into the functional diversity of the MAPK pathway. We sincerely appreciate your meticulous review of our study. The above explanations have been incorporated into the revised manuscript. Please see lines 375-384 of the revised version.

31. Line 217 – briefly describe the action of activators and inhibitors. Changes in protein levels of UGT356B1 are barely visible. It would be better to use a more sensitive assay to measure

RESPONSE: We sincerely appreciate pointing out this important issue. In the revised version, we have briefly described the action of activators and inhibitors in the appropriate section. Please see lines 228-229 of the revised version.

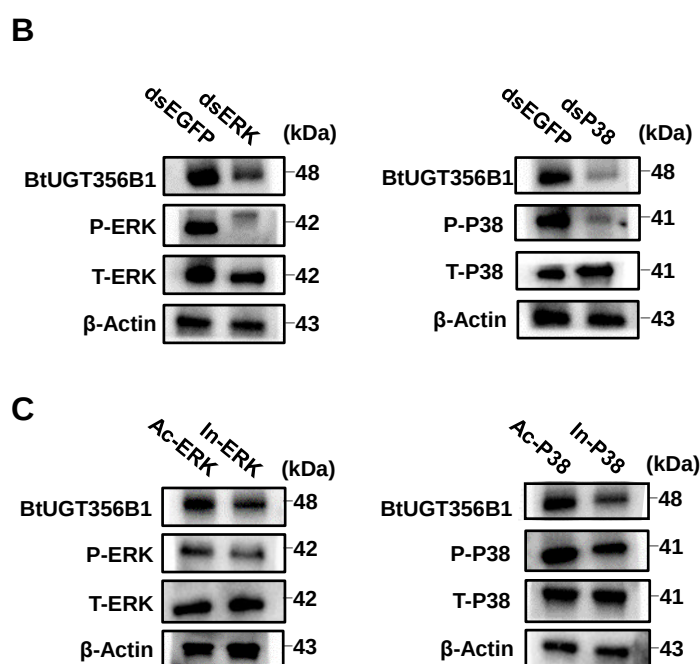
P38/ERK belong to the mitogen-activated protein kinase (MAPK) family within cells. Their activators primarily work by activating upstream kinases, triggering the phosphorylation of p38 or ERK, thereby initiating downstream signaling pathways to rapidly respond to intracellular and extracellular stimuli. Inhibitors, on the other hand, interrupt signal transmission by blocking the activity of upstream kinases or directly inhibiting the phosphorylation of p38/ERK. However, these activators and inhibitors mainly induce rapid phosphorylation cascades rather than the immediate accumulation of large

amounts of protein at downstream targets.

Regarding the almost undetectable change in BtUGT356B1 protein levels after p38/ERK inhibition, this may be attributed to the limited dynamic range of conventional target gene Western blot detection. Although the grayscale differences in the original images were subtle, adjusting the contrast and brightness (Response Figure1C) clearly revealed the fluorescence intensity disparity between the activators and inhibitors groups, confirming the protein degradation trend. Additionally, in the p38/ERK interference experiments, we observed a significant decrease in BtUGT356B1 protein levels (Response Figure 1B). Although the extent of BtUGT356B1 reduction after p38/ERK inhibition may vary depending on cell type and detection sensitivity, multiple independent experiments have confirmed its dependency.

In future studies, we will also follow the reviewers' suggestions to consider more sensitive detection methods for more accurate monitoring of target protein changes.

Response Figure1



Response Figure1. **(B)** Protein expression analysis of BtUGT356B1, Total-ERK (T-ERK), Total-P38 (T-P38), phosphorylated ERK (P-ERK), and phosphorylated P38 (P-P38) after ERK or P38 knockdown in the R^{#1} strain. **(C)** Expression of BtUGT356B1, Total-ERK, Total-P38, phosphorylated ERK, and phosphorylated P38 in the presence of activators or inhibitors of the ERK and P38 pathways. *β-actin* was used as a loading control. In-ERK, inhibitor of ERK; Ac-ERK, activator of ERK; In-p38, inhibitor of p38; Ac-p38, activator of p38.

32. Line 233 – quickly cite that the TF of *B.bemisia* were cloned.

RESPONSE: We sincerely appreciate the meticulous and detailed review. We

have cited relevant research literature here.

33. *Line 239-240 – quickly explain which essential part of HR39 are mutated in DN1 and DN2*

RESPONSE: We appreciate the question raised by the reviewer. We respectively constructed two functional domain deletion mutants of HR39: DN1(deletion of DNA binding domain (DBD, amino acid 326-418)) and DN2(deletion of ligand binding domain (LBD, amino acid 499-661)). Please see lines 248-250 of the revised version.

34. *Line 258 – change the title. As it is now it seems that HR39 was artificially overexpressed to study its function.*

RESPONSE: We appreciate this insightful comment. The title has been revised to avoid ambiguity: “HR39 is involved in the resistance of whitefly to TMX and IMI.”

DISCUSSION

Line 287 – I think the sentence should be changed into “... widely involved in the development of resistant in whiteflies.” Remove “whiteflies were no exception”

RESPONSE: Thank you for your meticulous review and your valuable suggestions. Regarding the suggestions for sentence modification you pointed out, I fully agree with your advice and have made corresponding adjustments. Please see Line 299-300 in the revised version.

Line 289 – plants?

RESPONSE: We apologize for the negligence here. The correct term should be [strains], which has been amended in the revised manuscript.

Line 293-295 – repetition.

RESPONSE: We have carefully revised the manuscript to eliminate redundant expressions. Please see Line 300-306 in the new version.

Line 297 – only some UGTs showed overexpression.

RESPONSE: We are grateful to the reviewers for the meticulous review of the language expression. We have modified this to "some of which".

Line 313-317 – the sentence is not clear, rephrase.

RESPONSE: We sincerely appreciate your valuable suggestions. Optimized sentence: "We observed an analogous resistance generation process in

transgenic *D. melanogaster*, consistent with the RNAi-based findings on *BtUGT356B1*-mediated detoxification of TMX and IMI." Please see Line 327-329 in the new version.

Line 320-322 – I don't understand the rationale behind this sentence: please argue in the manuscript on why you arrived to this conclusion.

RESPONSE: We thank the reviewer for raising this profound and critical point. We sincerely apologize for any lack of clarity in our presentation. Our conclusion is based on a synergistic analysis of direct metabolic experimental data and molecular docking results:

First, our data show that recombinant BtUGT356B1 can metabolize thiamethoxam (23.71%, $P = 0.006$) and imidacloprid (27.58%, $P < 0.0001$). This first confirms that BtUGT356B1 indeed possesses the ability to directly degrade these two neonicotinoid insecticides, which is the basis for its involvement in resistance.

However, the binding energy in molecular docking reflects catalytic efficiency: the lower the binding energy, the higher the enzyme-substrate affinity and catalytic potential. Our binding energy results show that BtUGT356B1's binding energies with imidacloprid and thiamethoxam are similar with those of other derivatives, suggesting that binding affinity itself may not be the dominant factor. If we relied solely on direct catalysis, we might expect significantly better (i.e., lower) binding energies with high-efficiency substrates (such as imidacloprid and thiamethoxam) to maximize catalytic efficiency. However, the observed similarity in binding energies indicates that the binding affinity of BtUGT356B1 to these insecticides is not the sole or dominant factor in its functional evolution.

Therefore, given that BtUGT356B1 possesses demonstrable direct metabolic activity but lacks corresponding specificity in binding characteristics, its contribution to the overall resistant phenotype likely extends beyond this direct metabolic capacity. This suggests that BtUGT356B1's contribution to resistance may extend beyond mere enzymatic catalysis—for instance, by synergizing with other genes to indirectly enhance overall resistance through modulation of cellular detoxification pathways. This hypothesis will be a key focus of our subsequent research.

Line 326- phase I and II are cited a bit out of the blue, although the explanation is provided just below. I would rephrase to first define what phase I and II are and then explain the rest. Still, what does it mean that your study suggest that UGT356B1 may not solely rely on direct catalytic ability? Why this conclusion? And the sentence below seems to be in contrast with this statement.

RESPONSE: We sincerely appreciate your meticulous review of the manuscript and valuable comments. The initial formulation may have lacked clarity. The revised version is as follows: "Phase I detoxification primarily involves oxidation,

reduction, or hydrolysis reactions mediated by the cytochrome P450 enzyme, which converts lipophilic toxins into water-soluble intermediate products. Phase II detoxification further modifies these products through conjugation reactions (e.g., glucuronidation), significantly enhancing their water solubility and reducing toxicity, thereby facilitating their elimination from the body.”

Our conclusion speculates that BtUGT356B1 may not only rely on the direct catalytic ability of metabolizing thiamethoxam and imidacloprid, due to the following reasons:

The recombinant BtUGT356B1 can directly metabolize thiamethoxam and imidacloprid, demonstrating that this gene enhances pesticide excretion efficiency through glycosylation. This confirms its glycosylation activity is associated with resistance, consistent with metabolic data, thereby validating BtUGT356B1's fundamental detoxification function.

Molecular docking reveals that UGT356B1's binding energy with imidacloprid and thiamethoxam is comparable to other derivatives, suggesting its catalytic efficiency is not specifically optimized for these insecticides. If resistance relied solely on direct metabolism, binding energy would significantly decrease to enhance catalytic efficiency—a deviation observed in the actual data.

If direct catalysis were the sole mechanism, we would expect substantially lower binding energy with high-efficiency substrates (e.g., imidacloprid and thiamethoxam). Thus, we hypothesize that UGT356B1's contribution to resistance may extend beyond enzymatic catalysis, potentially involving indirect mechanisms such as synergistic effects or regulation of other genes to amplify cellular detoxification pathways (Ref.1-2). This aligns with UGT356B1's proposed mechanism, collectively highlighting the multifunctionality of UGT family members in resistance.

In summary, we reasonably infer that *BtUGT356B1*'s role in resistance is more complex, with direct catalysis being one of the core mechanisms, while indirect effects expand its biological significance. We have supplemented relevant arguments in the Discussion and rephrased them to ensure academic rigor. Please see Line 334-336 in the new version.

References

- [1] Kaplanoglu, E., Chapman, P., Scott, I.M. & Donly, C. Overexpression of a cytochrome P450 and a UDP-glycosyltransferase is associated with imidacloprid resistance in the Colorado potato beetle, *Leptinotarsa decemlineata*. *Sci Rep.* **7**, 1762 (2017).
- [2] Bai B. et al. Different detoxification strategies of fruit borers adaptation to quercetin. *Pestic Biochem Physiol.* **215**, 106649(2025).

Line 343 – this has already been reported above.

RESPONSE: We sincerely appreciate the valuable suggestions. We have further integrated the discussions before and after the text and removed the redundant references at this point.

Line 345 – there is an hanging “t”.

RESPONSE: We appreciate the reviewer pointing out the oversight here. We have removed this redundant word from the revised version.

Line 349 – the example provided are not on neonicotinoids but on Bt toxins, so I would not start the sentence with “for example” as this are not really examples of the sentence above.

RESPONSE: We sincerely appreciate your identification of the inaccuracy in the citation of examples within the text. Your observation is entirely correct, as these two instances belong to distinct pesticide categories, and their misrepresentation would indeed lead to conceptual confusion. The revised version has been updated accordingly with the following modifications:

“The MAPK signaling pathway plays a significant role in development, metabolism and stress response⁵⁴. This study demonstrates that this pathway plays a key role in the resistance of whitefly to thiamethoxam and imidacloprid by regulating the UGT-mediated detoxification process. Recent studies have further revealed that the MAPK-CREB-CYP6CM1 signaling pathway mediates the broad-spectrum resistance of whitefly to neonicotinoid insecticides³³. The work of others also suggests that MAPK-dependent hormonal signaling in Bt resistance in *P. xylostella* ⁵⁵.”

Line 366 – domains abbreviation were already defined.

RESPONSE: We sincerely appreciate your rigorous and meticulous review. We have deleted the duplicate definitions and revised the sentence to read: “Nuclear receptors are key transcriptional regulators in metazoans. Their DBDs and LBDs typically cooperatively regulate target gene expression by interacting with promoter or enhancer sequences at specific recognition sites.”

Line 374 – another work on mites demonstrated that HR96 can regulate several detoxification enzymes: <https://www.nature.com/articles/s41467-023-40778-w>

RESPONSE: We sincerely appreciate for identifying this critical reference, which significantly strengthens the theoretical foundation of our study. The research confirms that the nuclear hormone receptor HR96 in *T. urticae* is the primary determinant of differential detoxification gene expression between resistant and susceptible strains, further supporting its central role in arthropod detoxification metabolism regulation. Please see Line 401-405 in the new version.

Line 380 – the concluding paragraph could be strengthened to more clearly summarize the main findings and their implications.

RESPONSE: We sincerely appreciate your review and valuable suggestions.

We have carefully considered your feedback and revised the final paragraph to strengthen the summary of key findings and their implications. The specific modifications are as follows: “In summary, the MAPK signaling pathway significantly promotes the overexpression of the *BtUGT356B1* by mediating the activation of the HR39 transcription factor, thereby enhancing the detoxification function of UGT enzymes. This mechanism has been identified as a key driver of metabolic resistance in *B. tabaci* to thiamethoxam and imidacloprid. The elucidated MAPK-HR39-*BtUGT356B1* signaling cascade deepens the understanding of the molecular mechanisms underlying neonicotinoid resistance at the transcriptional level and provides a novel theoretical framework for research on insect metabolic resistance.”

MATERIALS AND METHODS

35. Line 389 – names TMX-resistant and IMI resistant are confusing. I guess this is due to selection of the strains on those pesticides, however based on toxicity results there is cross-resistance. Furthermore it is necessary to indicate how many generation of selection occurred and the concentration of insecticides used for selection.

RESPONSE: We sincerely appreciate the valuable comment. The omission of information in the previous description may indeed cause confusion. We have revised and supplemented the following details (Lines 441-449):

“The *B. tabaci* MED strain was initially collected from tomato plants in Hangzhou, Zhejiang (30°20'N, 120°38'E) in 2011. Using the two-way method, this strain was divided into three batches with identical genetic backgrounds. Two batches were then used to construct laboratory-resistant strains (R^{#1} and R^{#2}) by selection with thiamethoxam and imidacloprid at a LC₅₀ concentration (100 mg/L), respectively. The remaining batch was used for diagnostic dose bioassays on the offspring of single-pair, non-intercrossed whiteflies to establish a laboratory-susceptible strain (S^{#1}). This susceptible strain has been maintained for over a decade without exposure to any insecticides.”

36. Line 408 – “total protein” indicate how protein extraction was executed.

RESPONSE: We thank the reviewer for pointing out the need for clarity regarding the protein extraction procedure used to obtain the “total protein” samples. We have now added the following detailed description to the Materials and Methods section: “Approximately 200 adults from each strain were collected to extract the total Protein using the ProteinExt Kit (Beyotime, China), and the protein concentration was quantified to 10µg according to the instructions of the BCA Protein Assay Kit (Beyotime, China).” Please see Line 464-467 in the new version.

37. Line 412 – “... second round of washing (5 times)” not clear. Was it washed five times?

RESPONSE: We sincerely appreciate the comment. We apologize for the lack of clarity in the original wording. To clarify, “a second round of washing (5 times)” refers to five separate washing steps, each performed with fresh buffer. The revised version is as follows: “The washing step was repeated five times (each with fresh buffer) before adding Chromogen Solutions A and B, followed by 10-min incubation at 37°C.”

38. Line 435 – “as well as”

RESPONSE: We sincerely appreciate the meticulous review. We sincerely apologize for these oversights and have corrected them in the revised version.

39. Line 475 – indicate PCR conditions or refer to Supp. tables.

RESPONSE: We sincerely appreciate the reviewer's valuable feedback on our manuscript. In response, we have supplemented the PCR reaction system and procedures in the Supplementary Data 2, with detailed information provided below.

Table 1 PCR reaction system

Component	Volume
GC Buffer	5 μ L
10 mM dNTPs	0.5 μ L
Forward primer(F)	1.25 μ L
Reverse primer(R)	1.25 μ L
cDNA	1 μ L
Q5 enzyme	0.25 μ L
ddH ₂ O	10.75 μ L

Table 2 PCR reaction procedure

Step	Temperature	Running time
1	98°C	30 s
2	98°C	10 s
3	55~60°C	30 s
4	72°C	30 s
5	Go to step 2	35 cycles
6	72°C	2 min
7	4°C	Forever

40. Line 548 – what does “conserved mutant forms” means?

RESPONSE: We sincerely appreciate your identification of unclear phrasing in our manuscript. In response, we have removed the potentially ambiguous “conserved”.

GENERAL COMMENTS

The authors should discuss and highlight why all the strains investigated (both field strains and artificially selected) seems to have evolved the same resistance mechanism.

I suggest to revise the entire manuscript for stylistic inconsistencies that might have not been indicated in my comments above.

I suggest to include in the results section a brief explanation of experimental procedure and rationale of experiments to improve readability. Above there are some specific comments addressing this but it should be applied more broadly.

RESPONSE: We sincerely appreciate your meticulous review and insightful comments. We fully agree that all *B. tabaci* strains, including laboratory and field strains, exhibit the same core resistance mechanism: MAPK-mediated HR39 activation, which promotes *BtUGT356B1* overexpression, enhances UGT enzyme detoxification activity, and is likely the primary cause of resistance to thiamethoxam and imidacloprid. We will now discuss and emphasize this phenomenon from the perspectives of evolutionary selective pressures, functional advantages of the mechanism, and consistency of experimental evidence. The supplementary content has been added and refined in the Discussion of the new version (Line 410-429). The additions are as follows:

“In natural environments, organisms often independently evolve identical adaptive traits when facing similar selective pressures²³. Notably, all whitefly strains in this study, including laboratory and field strains, exhibit a unified resistance mechanism: activation of transcription factors HR39 via the MAPK signaling pathway, ultimately enhancing UGT enzyme detoxification capacity. This finding strongly suggests that the pathway confers significant evolutionary advantages under selection pressures from neonicotinoid insecticides, potentially establishing it as a primary metabolic resistance mechanism. Firstly, as a classic stress-response pathway, MAPK rapidly senses chemical stress and initiates downstream gene expression⁶⁴. Unlike rare single-point mutations, this pathway allows modulation at multiple nodes, facilitating independent evolution across genetic backgrounds⁶⁵. Secondly, UGT enzymes, with their broad-spectrum potential in glycosylating xenobiotics, play a pivotal role in detoxification⁶⁶. Additionally, the independent recurrence of identical resistance phenotypes in laboratory strains mirrors those in field populations, providing robust experimental support. This not only rules out dominant confounding factors in complex field environments but also suggests high predictability in resistance evolution under similar chemical pressures. Therefore, we speculate that the independent and repetitive occurrence of this mechanism in the two types of strains is not accidental but the result of evolutionary pressure.

References

- [23] Xia, J.X. et al. Whitefly hijacks a plant detoxification gene that neutralizes plant toxins. *Cell*. **184**, 1693-1705 (2021).
- [64] Taouktsi, E. et al. Mitochondrial p38 Mitogen-Activated Protein Kinase, Insights into Its Regulation of and Role in LONP1-Deficient Nematodes. *Int J Mol Sci*. **24**(24),17209 (2023).
- [65] Cargnello, M. & Roux, P.P. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol. Mol. Biol. Rev.* **75**, 50-83 (2011).
- [66] Bock, K.W. The UDP-glycosyltransferase (UGT) superfamily expressed in humans, insects and plants, Animal-plant arms-race and co-evolution. *Biochem Pharmacol*. **99**,11-17 (2016).

To address the reviewers' valuable comments, we have revised the manuscript throughout, standardizing terminology, sentence structure, and figure annotations to ensure logical coherence. Specifically, we refined the transitions between the Results and Discussion sections to enhance narrative flow and supplemented additional critical experimental details in the Materials and Methods. All cited references were thoroughly re-evaluated and verified to ensure each accurately reflects the original study.

We are grateful for the reviewer's insightful feedback, which has been instrumental in improving the overall clarity and scholarly integrity of our manuscript.

FIGURES and figure caption.

Figure 1

A-B: IMI and TMX abbreviations are not used in the figure but are reported in figure caption. There is no color legend for dose-response curves.

C: is too small. Can become supp. figure.

RESPONSE:

Figure A-B: We have unified the expressions within the figures and in the captions, replacing both IMI and TMX in the captions with full names, imidacloprid and thiamethoxam. Clear color legends have been added to the dose-response curves in panels A and B.

Figure C: After careful consideration, we significantly increased the size of the original figure 1C, moved it to the supplementary materials, and recompiled it into supplementary Figure S1.

Figure2

A-B: Indicate dose of TMX in the figure or figure legend.

C: field strains: I think it is not correct to refer to R1 and R2 as field strains (as commented above).

Description of panel E and D is inverted.

RESPONSE:

Figure 2A-B: Thank you for your insightful comment. We have now clearly indicated the dose of thiamethoxam (TMX) used in both panels A and B. The specific concentrations (50 µg/mL) are now labeled on the figure legend.

Figure 2C: We sincerely apologize for the oversight. We have revised the figure legend to accurately describe them as "laboratory strains and field strains" to avoid any confusion.

Figure 2D-E: We sincerely thank the reviewers for identifying this labeling error. We have now swapped the labels for panels E and D in Figure 2 to ensure consistency with the figure caption and the main text.

Figure 4

Define abbreviations of Total-ERK (T-ERK) and phosphorylated ERK. The same for p38.

RESPONSE: We sincerely appreciate the reviewers' valuable suggestions. The abbreviations have been uniformly added to the figure captions.

Line 859 – which lane is activator and which one is inhibitor.

RESPONSE: We sincerely apologize for this oversight. The figure captions have been updated to clearly indicate the activator and inhibitor lanes.

Figure 5

Line 878 – define what activity.

RESPONSE: We thank the reviewers for pointing out the unclear phrasing. The figure legend has been revised as follows: "Dual-luciferase reporter assay of *BtUGT356B1*^(-174/-1) promoter with Wt1 (Mut1) or Wt2 (Mut2) and transcription factor HR39."

Mut1 sequence provided in panel F is wrong (=WT).

RESPONSE: We sincerely appreciate the reviewers' identification of the sequence annotation error, which was an oversight on our part. We have re-examined and corrected this error, and the accurate Mut1 sequence is now provided in Figure F.

*Figure 6D – I find this panel unclear. I would be consistent with the other panel/figures and use bar chart. Also the title "mortality of RNAi" is incorrect. Mortality of *B. tabaci* after RNAi treatment.*

RESPONSE: We sincerely appreciate your meticulous review of the visualization details. As requested, we have addressed the two key issues:

Unified all panels to bar charts (consistent with Figure 4's mortality data presentation for *B. tabaci* post-RNAi), enhancing clarity in mortality distribution.

Revised "RNAi mortality" to "mortality of *B. tabaci* after RNAi treatment" to

precisely align with experimental procedures.

These changes are now reflected in the revised version.

GRAPHICAL ABSTRACT

I think the “wi-fi” symbol should be inverted to show that MAPK cascades acts on ERK and p38. As it is now it seems to be the opposite.

RESPONSE: We sincerely appreciate your valuable feedback regarding the Graphical Abstract. In response to your suggestion, we have adjusted the orientation of the Wi-Fi symbol to an inverted position to more intuitively illustrate the activation signal transmission process along the ERK/p38 cascade pathway.

Reviewer #2 (Remarks to the Author):

The manuscript by Du et al investigated the source of metabolic resistance in field- collected population of Bemisia tabaci. The authors screened select Uridine diphosphate (UDP)-glycosyltransferases (UGTs) genes and identified increased in expression and biochemical activity of BtUGT356B1. Further investigation through gene knockdown via RNAi and qpCR indicated a collection between BtUGT356B1 expression and resistance to thiamethoxam and imidacloprid. Furthermore, in vivo and in vitro studies MAPKs P38 and ERK identified via the TF HR39 as the regulatory mechanism for BtUGT356B1.

The manuscript provides evidence of the role of metabolic resistance and potential regulatory mechanism. Before the manuscript is worthy of being considered for publication in this journal but the authors need to address the issues list below before been accepted.

RESPONSE: We sincerely appreciate the time and effort you have dedicated to reviewing our manuscript and for providing us with such valuable and insightful comments. In response to the questions you raised, we have carefully analyzed each one and completed the necessary revisions. Below is our point-by-point response.

1. The current structure of the manuscript indicates a fishing expedition approach on the link between BtUGT356B1 and resistance in B. tabaci. The authors made np attempt to elucidate the potential contribution of other source of resistance (P450s, GTS, esterase, ABC transporters and SNPs). Not addressing these other pathways to insecticide resistance can overstate the contribution of BtUGT356B1 to resistance.

RESPONSE: We sincerely appreciate your meticulous review and valuable suggestions. We fully agree with your point regarding the need to clarify the potential roles of other resistance mechanisms (such as P450s, GSTs, esterases, ABC transporters, and SNPs) to avoid overstating the contribution of BtUGT356B1. In fact, multiple studies have elucidated the molecular

mechanisms of insecticide resistance in *B. tabaci*. For example, the overexpression of the *CYP6CM1* gene is associated with imidacloprid resistance, and its upstream regulator, p38/CREB, also shows an upregulation trend (Ref.1). Similarly, the overexpression of *CYP4C64* in *B. tabaci* is a key contributor to neonicotinoid resistance, and a novel RNA methylation-mediated regulatory mechanism has been identified (Ref.2).

We acknowledge that insecticide resistance in insects is typically a complex trait mediated by multiple mechanisms. During the design phase of this study, we fully considered this context. Therefore, our research does not aim to comprehensively elucidate all resistance mechanisms in *B. tabaci* but instead focuses on investigating the specific role of the UGT family, particularly *BtUGT356B1*, in mediating resistance to specific insecticides, such as neonicotinoids.

We understand the term "fishing expedition" to imply broad exploration without a clear hypothesis. However, our study is based on the significant upregulation of *BtUGT356B1* in resistant strains, as indicated by prior transcriptomic data. Our experimental design including analysis of gene induction patterns, RNAi-mediated gene silencing and its impact on pest mortality, assessment of transgenic *D. melanogaster* sensitivity to insecticides, and validation of *BtUGT356B1*'s metabolic activity—was specifically designed to test the hypothesis that "*BtUGT356B1* is an important factor contributing to the resistant phenotype."

Regarding the other resistance pathways you mentioned, we fully agree that they may coexist and play a role. In the discussion of the manuscript, we have already acknowledged these possibilities and noted it as a limitation. We have strengthened this section to clarify that while our study primarily focuses on the contribution of the UGT metabolic pathway, it does not exclude the possibility of synergistic or independent roles of other metabolic enzymes (e.g., P450s, GSTs) or ABC transporters in the target population.

We have cited authoritative references on insect resistance, particularly the well-documented existence of multiple resistance mechanisms in *B. tabaci*, to contextualize our findings (the function of *BtUGT356B1*) within a complex resistance network. We have revised our statements to emphasize that our data demonstrate *BtUGT356B1* is an important and necessary factor in resistance formation, but not necessarily the sole one. Functional validation experiments (e.g., RNAi and metabolic assays) show that silencing this gene significantly increases susceptibility, and *BtUGT356B1* can directly metabolize two insecticides, directly proving its necessity, though we do not claim sufficiency.

We have added a future perspective in the conclusion, explicitly stating that future research should employ more comprehensive omics approaches to quantify the relative contributions of different resistance mechanisms to overall resistance and to elucidate their interactions.

In summary, we believe these textual revisions and clarifications will more accurately position the significance of our study: we have definitively

demonstrated that *BtUGT356B1* is a critical component of *B. tabaci*'s resistance mechanism, without negating the existence of other components. Thank you again for prompting us to make our arguments more rigorous and comprehensive.

References

- [1] Fu, B. et al. GPCR-MAPK signaling pathways underpin fitness trade-offs in whitefly. *Proc Natl Acad Sci U S A*. **121**, e2402407121 (2024).
- [2] Yang, J. et al. m6A-Mediated Regulation of *CYP417B1* Contributes to Imidacloprid Resistance in *Bemisia tabaci*. *J Agric Food Chem*. **73**, 16766-16777 (2025).

2. What about inductive and constitutive expression of BtUGT356B1 under exposure to THI and IMI.

RESPONSE: We sincerely appreciate your insightful comments and the attention you have given to the function of the UGT gene in our study. The question you raised about the expression pattern of *BtUGT356B1* under the action of thiamethoxam (THI) and imidacloprid (IMI) is crucial for elucidating the gene's function.

First, regarding constitutive expression (basal expression level), our data show that in the absence of insecticide stress, the susceptible strain of *B. tabaci* exhibits a low basal expression level of *BtUGT356B1* (Fig. 2A). This suggests that the gene is not a broadly expressed housekeeping gene, and its function may be more inclined to be activated under specific stress conditions.

Second, and more importantly, regarding induced expression. After subjecting *B. tabaci* to sublethal concentrations of TMX and IMI, respectively, we found through qPCR and Western blot analyses that the expression level of *BtUGT356B1* was significantly and time-dependently upregulated. Under TMX treatment, the expression of *BtUGT356B1* was significantly higher than the control at both 6 and 12 hours, peaking at 12 hours with an induction fold exceeding 2.5 (Fig. 2A).

Similarly, under IMI treatment, we observed a comparable induced expression trend, with a similar upregulation pattern to the TMX treatment group. This suggests that this induction response may have some generality across neonicotinoid insecticides.

These results strongly indicate that *BtUGT356B1* is a metabolic enzyme gene that can be induced by the neonicotinoid insecticides THI and IMI, rather than a static gene.

3. It is surprising that the authors conducted no genome scan or transcriptomic analysis to have a comprehensive understanding of the mechanism of the genetic basis of resistance in the Bemisia populations.

RESPONSE: We fully acknowledge the critical importance of employing

genomic or transcriptomic approaches for systematic screening and analysis, as you rightly pointed out. These methodologies are indeed essential for revealing the full spectrum of genetic variations and global gene expression changes that may occur in populations during resistance evolution, which is crucial for a comprehensive understanding of whitefly resistance mechanisms.

However, our study specifically focused on the UGT gene family and conducted in-depth functional investigations of *BtUGT356B1*, primarily because our preliminary experiments and literature review led us to formulate a well-defined scientific hypothesis: the UGT-mediated detoxification pathway likely plays a pivotal role in whitefly resistance to neonicotinoid insecticides. Consequently, our research design did not adopt an omics screening approach but instead followed a hypothesis-driven research pathway. We first conducted targeted screening of the UGT gene family and clearly demonstrated a direct correlation between *BtUGT356B1* expression levels and resistance to thiamethoxam and imidacloprid through experiments measuring UGT enzyme activity, qPCR, RNAi knockdown, and insecticide metabolism assays. We then delved deeper into the mechanistic level, not only confirming the detoxification function of *BtUGT356B1* but also revealing its upstream transcriptional regulatory mechanism - specifically, the regulation of *BtUGT356B1* by the transcription factor HR39 through the MAPK signaling pathways (P38/ERK). This discovery, from the "key gene" to the "regulatory pathway," represents the core innovative value of our study.

Naturally, we recognize that while our study has revealed a specific gene pathway, it has not fully characterized the genetic basis of whitefly populations. This limitation not only defines the scope of our current work but also points to important directions for future research. We plan to conduct whole-transcriptome sequencing (RNA-seq) on multiple geographically distinct whitefly populations with varying resistance levels in subsequent studies. Through comparative transcriptomic analysis, we aim to systematically identify other key genes and pathways (such as other UGT members, P450s, ABC transporters, etc.) that are commonly differentially expressed in resistant populations, thereby constructing a more comprehensive molecular mechanism of whitefly resistance.

In summary, the primary objective of this study was to conduct an in-depth analysis of a specific pathway rather than a broad screening of all possible mechanisms. We are confident that the current study has made valuable new contributions to the field of whitefly resistance, and your insightful suggestions have provided crucial guidance for the deepening of our future research.

4. What is the rationale for selecting the candidate UGT genes that were screened to identify BtUGT356B1.

RESPONSE: We sincerely appreciate your insightful question regarding the selection rationale and identification methodology of UGT genes in our study. The fundamental principle underlying our screening of candidate UGT genes to

identify BtUGT356B1 involved analyzing the differential expression and functional characteristics of UGT family members under specific conditions, integrating bioinformatics predictions with experimental validation to ultimately pinpoint the target gene.

The selection of UGT genes adhered to the following criteria:

- Differential expression: RNA-seq data revealed that these genes exhibited significantly higher expression levels in the resistant strain compared to the susceptible strain.
- Functional homology: These genes demonstrated homology with insect UGTs known to participate in pesticide metabolism.
- Genomic context: Within the *B. tabaci* genome, these genes were located in regions previously identified as detoxification enzyme gene clusters.

For the identification of candidate UGTs, we employed previously published transcriptome data (Accession No.: GSE42102) (Ref.1), which is publicly available in the NCBI database. The genomic structures of UGT genes and BLAST analyses were sourced from NCBI. Full-length cDNA sequences were derived from the transcriptome data and validated via qPCR, while phylogenetic trees were constructed for functional clustering.

As detailed in the supplementary materials (Supplementary Figure 2), the genomic positions, transcript lengths, and domain architectures of each UGT gene are comprehensively documented. This dual evidence from expression profiling and evolutionary positioning provides a preliminary basis for prioritizing *BtUGT356B1* as a candidate for further investigation.

References

[1] Yang, N. et al. Transcriptomic and proteomic responses of sweet potato whitefly, *Bemisia tabaci*, to thiamethoxam. *PloS one*, **8**, e61820(2013).

5. The abstract and introduction can be improved with better transition and connection between pesticide usage and resistance and the need to connect regulatory mechanism to source of resistance.

RESPONSE: We sincerely thank you for your valuable suggestions on this manuscript. Based on your suggestions, we have comprehensively revised the abstract and preface, with a focus on strengthening the connection between pesticide use and metabolic resistance, and highlighting the association between regulatory mechanisms and the sources of resistance.

In the abstract and the first paragraph of the Introduction, we clearly pointed out that the excessive/abusive use of pesticides is the core driving force for the evolution of metabolic resistance, and added the transcriptional regulatory mechanisms involved in metabolic resistance (see line 17-21 in the revised version). Before the sources of resistance (enhanced metabolic detoxification) in the preface section, the regulatory mechanism has been added, and its role as a bridge connecting pesticide use and metabolic resistance phenotypes has become increasingly prominent (see line 41-48 and

line 62-63 in the revised version).

6. Line 94: can the authors qualify the strength of UGT's contribution to resistance in this study.

RESPONSE: We sincerely appreciate the reviewer for raising this profound question, which is crucial for elucidating the specific contribution of resistance mechanisms. We fully agree that accurately assessing the contribution intensity of the UGT family in mediating *B. tabaci* resistance to neonicotinoid insecticides is a critical step in deciphering its resistance mechanism.

Regrettably, at this stage of our study, we are unable to provide a precise quantitative value for the contribution intensity. This is primarily because the resistance phenotype in field populations typically results from the synergistic or additive effects of multiple mechanisms. However, to address your concern, we have emphasized the following points in the Discussion of the manuscript (Line 356-365), which indirectly demonstrates and relatively evaluates the contribution intensity of UGT.

First, by treating resistant strains with a UGT inhibitor and then testing their sensitivity to two insecticides, we found that the mortality of the resistant strain significantly increased, with its LC₅₀ values reverting toward the levels of the susceptible strain. This chemical synergism functionally confirms that UGT enzymes play an indispensable role in resistance (Fig. 1A-B). Additionally, the data show that in multiple resistant strains, the fold-change upregulation of the *BtUGT356B1* gene expression is significantly positively correlated with the resistance level to the insecticides (Fig. 2E). This result strongly suggests that the overexpression of these UGT genes is a significant contributing factor to the resistant phenotype. Furthermore, by expressing and purifying the candidate BtUGT356B1 protein in vitro, we conducted insecticide metabolism experiments. The results demonstrated that, compared to the control, the recombinant BtUGT356B1 protein significantly accelerated the metabolism of the insecticides, directly proving that UGT has the ability to metabolize thiamethoxam and imidacloprid.

In summary, our study systematically evaluates the contribution of UGT through the above experiments. While an absolute quantitative value is currently challenging to provide under the existing technical framework, our findings clearly indicate that the UGT family, particularly its highly expressed individual subtypes, plays a pivotal role in the development of *B. tabaci* resistance to neonicotinoids.

7. How sure are the authors that UGT is the sole source of resistance (monogenic vs polygenic)

RESPONSE: We sincerely thank the reviewer for raising this critical question. We fully agree that in complex biological systems, it is extremely rare for a single gene to be the "sole" source of a particular phenotype. The purpose and

conclusions of our study were never intended to prove that the UGT gene is the "only" source of whitefly resistance, but rather to confirm that it plays a central or primary driving role within the polygenic basis of resistance.

Our conclusion is based on multifaceted evidence, which collectively points to the central role of UGT while not excluding the potential synergistic contributions of other genes. Our research began with transcriptomic analysis of the resistant strain, where the UGT gene family emerged as one of the most significantly upregulated modules in the differential expression network. This itself indicates that resistance is a polygenic process.

In functional validation, RNAi-mediated knockdown of BtUGT356B1 significantly reversed the resistance phenotype, but this did not restore the population to full susceptibility. This result precisely demonstrates that UGT overexpression is necessary to achieve the current level of resistance, though it may not be sufficient, as other contributing genes (e.g., additional metabolic enzymes) may be involved. The substantial synergistic effect observed with UGT inhibitors further underscores the functional centrality of the UGT pathway. If an entirely independent and equally potent resistance mechanism unrelated to UGT existed, inhibiting UGT would not produce such a dramatic phenotypic reversal.

Thus, we are highly confident that UGT is not the "only" source, but we have robust evidence that the UGT-mediated metabolic detoxification pathway is a key mechanism driving resistance in the whitefly population studied.

We have further emphasized this point in the discussion section of the manuscript, explicitly stating that future research should employ more refined genetic tools to ultimately dissect the precise contribution of UGT in a multi-genic context. Once again, we sincerely appreciate your feedback, as it has made our arguments more rigorous and clear.

8. Provide the R² values for figures 2A and 2B.

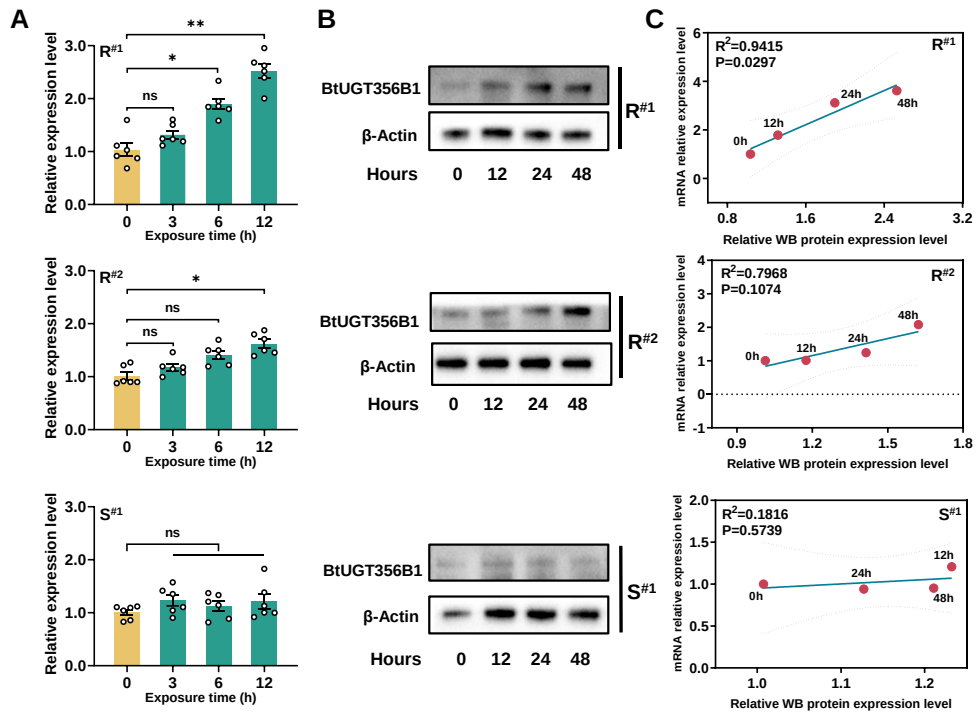
RESPONSE: The R² values for the correlation between mRNA expression (Figure 2A) and WB protein expression (Response Figure 2) are as follows:

For R^{#1}: R² = 0.9415

For R^{#2}: R² = 0.7968

For S^{#1}: R² = 0.1816

Response Figure 2



Response Figure2. **(A-B)** Relative mRNA expression levels and BtUGT356B1 proteins in susceptible and resistant strains after exposure to TMX and IMI at a concentration of 50 mg L⁻¹ (qPCR: $n = 6$, mean $\pm$ SEM; * $p < 0.05$, ** $p < 0.01$, Student's t -test). **(C)** Linear regression analysis of the mRNA relative expression level of *BtUGT356B1* and the expression of protein after induction.

Reviewer' comments:

Reviewer #1 (Remarks to the Author):

The authors put substantial effort in addressing the comments. However, although the clarity of the manuscript improved, several aspects need further clarification.

RESPONSE: We sincerely thank the editor and all reviewers for their insightful and constructive comments, which have provided crucial guidance and strong support for us to improve the quality of our manuscript. Below, the reviewers' comments are reproduced in italics and numbered for clarity, followed by our point-by-point responses marked in blue.

1. *"Regulatory mechanisms, as a bridge linking pesticide use and metabolic resistance phenotypes, are playing an increasingly prominent role." This sentence is not clear.*

RESPONSE: Thank you very much for your meticulous review and valuable suggestions! The ambiguity in the original phrasing you highlighted is indeed present. To more precisely clarify the role of regulatory mechanisms in linking pesticide exposure to metabolic resistance phenotypes, we have revised the manuscript as follows (Lines 62-69):

"During the development of metabolic resistance in pests, molecular mechanisms such as transcription factor regulation serve as a central link between pesticide exposure and resistance phenotypes. By modulating the expression of detoxification enzymes, these mechanisms can significantly enhance the metabolic and detoxification of pests. In recent years, their regulatory role has become increasingly prominent in the evolutionary process of resistance, emerging as a crucial factor driving the development of pest resistance."

2. *Line 71: "and promoting " is in italics.*

RESPONSE: We sincerely apologize for the oversight regarding the italic usage, and we have carefully reviewed and corrected the formatting accordingly.

3. *Titles in the Results section are inconsistent: some summarize what was done, while others highlight the main findings. They should be standardized for clarity.*

RESPONSE: We sincerely appreciate your meticulous review and valuable suggestions. We have carefully reviewed and standardized the inconsistent heading styles in the Results. Previously, some headings focused on describing the research content (e.g., "Activity of UGT and Synergism of 5-Nitrouracil and Sulfinpyrazone"), while others emphasized research conclusions (e.g., "HR39

Directly Upregulates BtUGT356B1 Transcription"), which indeed led to a lack of stylistic consistency. To enhance the clarity and professionalism, we have revised some headings in the Results to clearly stating the core findings of each subsection. The revised titles are as follows:

- (1) Original title: Activity of UGT and synergism of 5-Nitrouracil and Sulfinpyrazone
Revised title: Increased UGT activity and overexpression of UGTs are involved in whitefly resistance to TMX and IMI
- (2) Original title: *BtUGT356B1* Involvement in TMX and IMI Resistance
Revised title: *BtUGT356B1* mediates whitefly resistance to TMX and IMI
- (3) Original title: The binding of BtUGT356B1 to insecticides was analyzed by molecular docking
Revised title: BtUGT356B1 exhibits differential high-affinity binding to TMX and IMI
- (4) Original title: Metabolism of BtUGT356B1 on TMX and IMI
Revised title: Recombinant BtUGT356B1 protein directly metabolizes TMX and IMI

We have updated the revised headings to their corresponding positions in the main text and ensured that all result section headings throughout the manuscript consistently adopt the conclusion-driven style.

4. Line 121: "10 candidate UGT " specify here that this is based on a previous study.

RESPONSE: Thank you very much for your valuable suggestion. We have revised the manuscript in accordance with your comments. Specifically, we have added a clarification at the mention of "10 candidate UGTs" that these genes were selected based on our team's previous study (Yang, N. et al. Transcriptomic and proteomic responses of sweet potato whitefly, *Bemisia tabaci*, to thiamethoxam. PloS one, 8(5), e61820 (2013)), which confirmed the critical role of these UGTs in insecticide resistance of *B. tabaci* through transcriptomic analysis. The revised manuscript is as follows (Lines 127-130):

"The mRNA transcripts of 10 candidate UGT genes were measured using qRT-PCR in all strains. These UGTs were selected based on our team's previous study³⁴, where transcriptomic analysis confirmed their critical role in mediating thiamethoxam resistance in *B. tabaci*. "

Additionally, we have ensured the full citation of this prior study is properly included in the reference section of the manuscript.

5. Lines 142-145: A major concern is how 50 mg/L would affect the S strain before RNA extraction, as this concentration is considerably above the LC50

for the S strain (i.e., not sublethal). I understand that LC50s were measured after 24 h, while expression was measured earlier; however, mortality would still be expected in S (or at least the insects should be severely affected). Was mortality observed in the S strain? Were RNA extractions performed only from surviving individuals? Furthermore, these data indicate not only that basal BtUGT356B1 expression is higher in R strains, but also that R individuals can induce further expression when exposed to the pesticides. This distinction should be clearly stated in the Results. The lack of inducible expression in the S strain is interesting however it is probably due to the high dosage (e.g., individuals were severely affected and the endogenous response was inhibited)?

RESPONSE: We sincerely appreciate your highly valuable comments, which are essential for refining our study. Below are detailed responses to your questions:

(1) Survival status of the S^{#1} strain at 50 mg/L and RNA extraction subjects

It should be clarified that the LC₅₀ in this study was determined based on 48-hour mortality observation (see Materials and Methods, Line 546: "Mortality was recorded after 48 hours, and control mortality was required to be below 10% in each experiment."), whereas RNA extraction for induction experiments was conducted at 3, 6, and 12 hours post-insecticide treatment. Regarding the survival of the S^{#1} strain at 50 mg/L, most individuals remained alive after 12 hours of exposure, although some exhibited toxic symptoms such as slowed movement and appendage tremors. During RNA extraction, only surviving individuals in good physiological condition were selected. We have now explicitly addressed in the Materials and Methods section (Lines 596–598): "All adult whiteflies used for RNA extraction were strictly screened, and only those individuals that remained alive and in good physiological condition after insecticide treatment were selected. "

(2) Gene expression differences between resistant and susceptible strains

Your observation that the resistant strain not only exhibits a higher basal expression level but also further induces *BtUGT356B1* expression upon insecticide exposure, while the S strain shows no such response, is extremely accurate. We have added the following explicit statement in the Results (Lines 172-179): "Compared with the S^{#1} strain, the basal expression level of *BtUGT356B1* in the resistant strain was significantly upregulated. Moreover, after being treated with insecticides, the expression of *BtUGT356B1* in the resistant strain further increased, showing obvious induced expression characteristics. However, under the same treatment, there was no significant change in the gene expression of the sensitive strain. These results indicate that not only is the basal expression level of *BtUGT356B1* higher in resistant strain, but also the resistant strain can induce further expression after exposure

to insecticides. "

(3) Analysis of the Reason for the Lack of Induced Expression in the S^{#1}

Your speculation that the lack of induced expression in the S strain may be due to severe damage to individuals caused by high doses and the suppression of endogenous responses is consistent with our experimental observations and subsequent analyses. We have added the relevant analysis in the Discussion (Lines 353-359): " Interestingly, the susceptible strain did not exhibit induced expression under 50 mg/L concentration stress, which may be related to this concentration being close to its short-term lethal threshold. High-dose insecticides may directly damage the physiological functions of whiteflies, inhibit their normal stress response pathways, and prevent the effective activation of gene expression regulatory mechanisms."

We would like to express our sincere gratitude again for your meticulous review and professional suggestions. These revisions have significantly enhanced the scientific rigor and soundness of our paper.

6. Lines 145-146: In Figure 2B, UGT356B1 protein levels at time point 0 are very similar in S and R strains. The actin loading control suggests that this is not due to differences in total protein loaded. Why is this the case? This seems to contrast with Figure 2F, where UGT356B1 in S appears clearly lower than in R strains.

RESPONSE: We sincerely appreciate you raising the issue regarding the striking similarity in BtUGT356B1 protein levels between the S and R strains at time point 0 in Figure 2B, as well as the seemingly inconsistency with the results presented in Figure 2F. We would like to offer the following explanations and clarifications:

(1) Figure 2B is intended to illustrate the temporal changes in BtUGT356B1 protein levels under insecticide exposure. At time point 0, prior to insecticide exposure, we observed similar BtUGT356B1 protein levels in the S and R strains. This could be attributed to comparable basal expression levels of BtUGT356B1 among different strains under unstressed conditions, or it might be due to experimental errors. We employed β -actin as a loading control to ensure consistent protein loading, thereby eliminating the influence of loading discrepancies on the results.

(2) Figure 2F displays the basal expression levels of BtUGT356B1 protein across different strains under unstressed conditions. Here, we observed that the BtUGT356B1 expression level in the S strain is significantly lower than that in the R strain. This result does not contradict the observation made at time point 0 in Figure 2B, as Figure 2B depicts a comparison at a single time point, whereas Figure 2F simultaneously displays samples from multiple strains,

offering a broader comparison of basal levels. In brief, the similarity at time point 0 in Figure 2B reflects the basal expression state, while the disparities in Figure 2F underscore the differences in BtUGT356B1 protein expression among resistant, susceptible, and field-collected strains.

Although we have made every effort to maintain uniform experimental conditions, minor discrepancies may still exist between different experimental batches. The data shown in Figure 2B and Figure 2F pertain to different experimental batches and distinct sample sets. Consequently, variations in the expression levels of BtUGT356B1 protein may arise. We comprehend the reviewers' concerns and acknowledge that consistency in results is crucial in scientific research. In future studies, we will delve deeper into the variations in BtUGT356B1 expression under diverse experimental conditions and conduct comparative analyses among different strains. This will facilitate a more holistic understanding of the expression patterns and regulatory mechanisms of BtUGT356B1.

7. Section "MAPK Signaling Pathway Regulates BtUGT356B1 Transcription": I understand that phosphorylated ERK represents the active form of the protein. What may be confusing is that RNAi targeting ERK appears to affect only the phosphorylated (active) form, while total ERK protein levels remain unchanged. This outcome is biologically plausible, as ERK kinase activity and phosphorylation are not strictly dependent on total ERK abundance in a highly dynamic signaling system. RNAi preferentially impact newly synthesized, signaling-competent ERK, thereby reducing the active fraction without measurably altering total protein levels if they are high. This distinction should be explicitly addressed in the Discussion (now I believe it is not clear enough), and the discrepancy between total ERK and phosphorylated ERK should also be clearly described in the Results section (not indicated in the current version). Furthermore expression data of ERK would be a welcome addition as they would prove the effective downregulation of the gene. This was done for both RNAi experiment targeting UGT356B1 and HR39. This section should be rewritten including a more detailed analysis of the results. Furthermore, in Figure 4D, the S strain shows phosphorylated ERK comparable to resistant strains, while total ERK protein is considerably lower. This appears to contradict the explanation that phosphorylated ERK reflects ERK activity. Is this possible? Since phosphorylated ERK is a subset of total ERK, how can it be higher than total ERK? These observations should be discussed and clarified; otherwise, the protein data appear confusing.

RESPONSE: Thank you for your meticulous review of this manuscript and the invaluable comments provided. In response to your queries regarding ERK and phosphorylated ERK, we have conducted in-depth analyses and discussions. Below is our reply:

(1) Regarding the differences between total ERK and phosphorylated ERK:

We have added descriptions of the disparities between total ERK and phosphorylated ERK in the "Results" section and provided further explanations in the "Discussion" section. We emphasize that within a highly dynamic signaling system, the kinase activity and phosphorylation status of ERK are not strictly dependent on the abundance of total ERK. RNAi may preferentially affect newly synthesized ERK with signaling capability, thereby reducing the proportion of the active fraction without impacting the total ERK protein levels.

Revised Results (Lines 256-266): "Further western blot analysis revealed the expression levels of total ERK (T-ERK) and total P38 (T-P38) showed no significant fluctuations across the dsEGFP, dsERK, dsP38, and other treatment groups, with the band gray values remaining relatively stable. This indicates that the total expression levels of these two proteins are in a relatively constant state and are not significantly affected by the experimental treatments. In contrast, the expression levels of phosphorylated ERK (P-ERK) and phosphorylated P38 (P-P38) exhibited marked changes in response to different treatment conditions. For instance, the expression of P-ERK was significantly downregulated in the dsERK treatment group, while the expression of P-P38 was notably reduced in the dsP38 treatment group (Fig. 4C). "

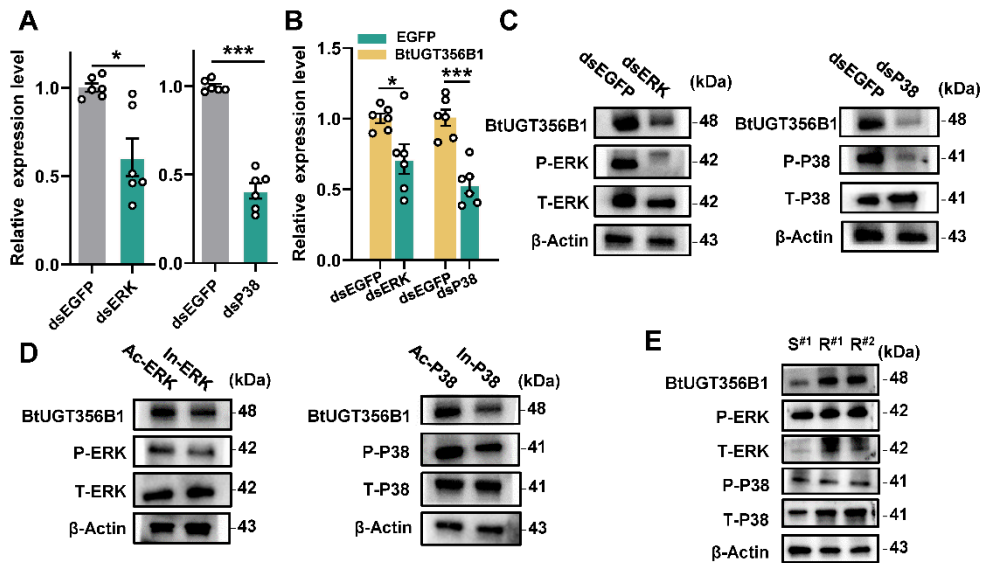
Revised Discussion (Lines 430-440): "We found that under treatment with dsRNA, activator or inhibitor, the total protein levels of ERK and P38 in whitefly remain stable, while their phosphorylation levels show significant dynamic changes. This is because in a highly dynamic signaling system, ERK kinase activity and phosphorylation do not strictly depend on the total abundance of ERK. RNAi preferentially targets newly synthesized, signaling-competent ERK, thereby reducing the active fraction without substantially altering total protein levels, which is consistent with the characteristic of the MAPK pathway that achieves rapid signal transduction through post-translational modification, and the phosphorylation levels of ERK and P38 can be regulated independently of their total protein levels. "

(2) Regarding the impact of RNAi on ERK/P38

We have supplemented the ERK/P38 expression data in the manuscript to validate the effectiveness of the RNAi experiments (Figure 4A). The results show that RNAi significantly reduced ERK/P38 expression levels, thereby supporting our conclusions on ERK function.

The revised Results is as follows (Lines 250-256): "To investigate whether another detoxification gene *BtUGT356B1* could be similarly regulated, we knocked down ERK and P38 in R^{#1} strain via RNAi. This approach successfully reduced the expression levels of ERK and P38 by 39.5% ($P = 0.013$) and 59.2% ($P = 1.462 \times 10^{-5}$) (Fig. 4A), respectively, which in turn led to a 0.29-fold ($P =$

0.026) and 0.47-fold ($P = 1.60 \times 10^{-4}$) decrease in *BtUGT356B1* expression (Fig. 4B). "



(3) Regarding the phosphorylated ERK level of S^{#1} strain

Under the basal state without pesticide induction, the phosphorylation level of ERK in the susceptible strain and the resistant strain is comparable, but the total ERK protein level in the S^{#1} strain is lower. This phenomenon is likely to occur, and the core reason lies in the fact that the detection of phosphorylated ERK is a specific recognition of the activated protein, and its signal intensity does not completely depend on the absolute content of total ERK. ERK phosphorylation is a rapidly responsive regulatory mode in cell signaling pathways. Even if the content of total ERK protein is low, as long as the cell is in a specific signal-activated state (such as low-level self-activation in the basal state), the limited ERK protein can be phosphorylated in a short time, thereby producing a phosphorylation signal comparable to that of cells with high total ERK content. Simply put, a small amount of ERK is highly activated, and its phosphorylation signal can be equivalent to that of a large amount of unactivated ERK. Secondly, in Western blot, if the antibody against P-ERK has higher affinity, or the signal amplification efficiency during detection is stronger, even if the actual content of P-ERK is slightly lower than that of total ERK, it may show a similar or even higher band signal.

We verified the phosphorylation regulatory mechanisms of ERK and P38 through experiments involving RNAi, treatment with activators (Ac-ERK, Ac-P38) and inhibitors (In-ERK, In-P38). After treatment with dsRNA, ERK/P38 activators or inhibitors, the total protein levels of ERK and P38 remained stable, indicating that our experimental treatments did not affect the gene expression or protein degradation processes of ERK and P38, but only specifically

regulated their phosphorylation modification states. In contrast to the stable total protein levels, the phosphorylation levels of ERK and P38 changed significantly after the above treatments. These results fully demonstrate that the phosphorylation levels of ERK and P38 can be regulated independently of their total protein levels, and phosphorylation modification is a reliable indicator reflecting their activities.

We have added this discussion in the revised manuscript to more clearly elucidate the phosphorylation regulatory mechanisms of ERK and P38. The specific revisions are as follows (Lines 440-451): "Although the ERK phosphorylation levels of the susceptible strain and the resistant strain are comparable in the basal state without insecticide induction, the total ERK protein level of the S^{#1} strain is lower. This may be because the detection of phosphorylated ERK is a specific recognition of activated proteins, and its signal intensity does not completely depend on the absolute content of total ERK. Simply, a small amount of ERK is highly activated, and its phosphorylation signal can be equivalent to that of a large amount of unactivated ERK. As a key intracellular signal amplifier, the MAPK pathway can rapidly regulate signal transduction without altering total protein levels, solely through switching phosphorylation modification states⁵⁴. Furthermore, we confirmed that ERK/P38 plays a key role in UGT-mediated resistance of whitefly to neonicotinoid insecticides."

To further strengthen the reliability of our conclusions, we plan to use phosphorylation site-specific antibodies in future studies to detect the exact phosphorylation sites to confirm whether the basal phosphorylation sites of P-ERK in the S strain are normal and to rule out potential interference from non-specific phosphorylation. Thank you again for your valuable professional insights, which have clearly guided the direction of our future research.

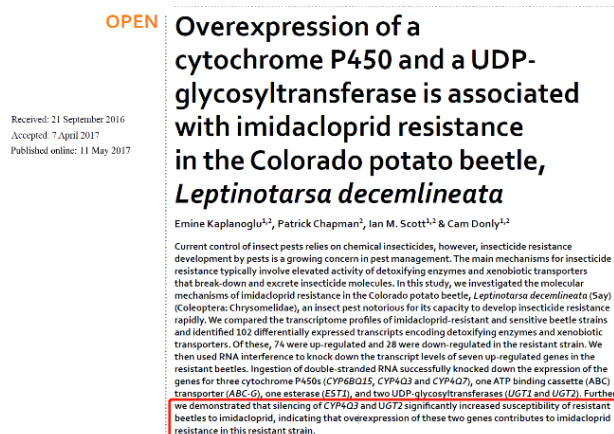
8. Line 245: *"was" seems out of place*

RESPONSE: We sincerely appreciate your meticulous review. We did indeed make a grammatical mistake at this point. To express our original intent more clearly and accurately, we have revised the sentence to (Lines 283-285): "only HR39 and HR78 showed significant increases in activity, with the mRNA level of HR39 being most upregulated in the resistant strains."

9. Line 312: *UGT2? Is there something missing?*

RESPONSE: Thank you for your meticulous and professional review. Here, "UGT2" refers to a specific gene named by the authors in this literature. It is systematically and clearly introduced in the methodology section (such as candidate gene validation), results section (RNAi target selection), and discussion section of the paper, and its significant impact on IMI resistance is ultimately confirmed through experiments.

Hereby, we attach the abstract of the relevant literature for the reviewer's verification:



10. Line 321-329: I would integrate what you described in your response about the low baseline expression. Mortality was already very high and does not provide strong evidence.

RESPONSE: We sincerely appreciate your meticulous review and invaluable comments on our manuscript.

In the revised manuscript, we have supplemented and improved the result interpretation section, and added discussion on the mechanism linking gene expression levels to phenotypic changes, enhancing the persuasiveness of our result interpretation.

Revised Results (lines 188-191): "In S^{#1} strain, although the change in target gene mRNA levels after dsRNA interference was not statistically significant (Fig. 2G), an increased mortality of whiteflies was observed under different insecticide treatments (TMX mg L⁻¹, IMI 100 mg L⁻¹) compared to the dsEGFP group (Fig. 2H-2I). "

Revised Discussion (lines 377-389): "In susceptible strain, although there was no significant change in the mRNA level of *BtUGT356B1* after dsRNA interference, the mortality of whiteflies still increased compared with the dsEGFP group. This might be that the baseline expression levels of *BtUGT356B1* mRNA and protein in susceptible strain are significantly lower than those in resistant strain. Such low expression makes the relative decrease after RNAi treatment less pronounced. Minor changes are more susceptible to technical variations, resulting in no significant difference in mRNA levels. Additionally, although the change in the mRNA level of *BtUGT356B1* in S^{#1} strain was not significant, an elevated mortality rate in whiteflies was observed after interference, indicating partial inhibition of *BtUGT356B1* function and a consequent reduction in insecticide tolerance. This further underscores the role of *BtUGT356B1* in neonicotinoid resistance. "

These supplementary explanations further confirm that *BtUGT356B1* is an

important functional gene involved in the formation and maintenance of neonicotinoid resistance in whiteflies.

11. *Line 332-336: This part remains unclear. From your response, it appears that you assume that if resistance relied mainly on direct catalysis, TMX and IMI should show much stronger binding than other substrates. However, detox enzymes are generally generalists and can metabolize several substrates. Catalytic activity is not solely determined by binding affinity. What can safely be stated is that BtUGT356B1 can bind TMX and IMI, and no obvious structural specialization is apparent. Furthermore, the sentence "It may also indirectly enhance overall resistance through synergistic effects or by regulating other genes to influence cellular detoxification pathways " seems disconnected from the docking analysis and is not entirely correct. What does it mean that UGT356B1 can regulate other genes? I suggest removing this sentence. Possible synergism with other detoxification enzymes, such as P450s or CCEs, is already discussed elsewhere.*

RESPONSE: We sincerely appreciate you pointing out that our interpretation of the potential functions of BtUGT356B1 beyond direct catalytic detoxification was not sufficiently cautious. We fully agree with your view and have streamlined and revised the relevant discussion, removing the over-interpreted parts (Lines 392-400):

" To clarify the function of BtUGT356B1 and its detoxification effect on TMX and IMI, we conducted molecular docking and in vitro metabolism studies. Molecular docking showed that the binding affinity of BtUGT356B1 with TMX and IMI was at the same level as that with other known substrates, which provides a structural possibility for the interaction between BtUGT356B1 and these two insecticides. In vitro metabolism studies directly confirmed that the recombinant BtUGT356B1 has ability to directly metabolize TMX and IMI, indicating that BtUGT356B1 can participate in the insecticide resistance process of whiteflies through direct catalytic detoxification."

12. *Line 358-360: "but" should be removed. "especially the highly expressed individual subtypes..." your data only validate a single UGT. I would stick to that without adding speculations about other UGTs.*

RESPONSE: Thank you for your meticulous review. We have deleted "but" and revised the sentence to read as follows (Lines 420-423):

"Although the resistance phenotype in field populations typically results from the synergistic action or accumulation of multiple mechanisms^{52,53}, our research conclusion clearly indicates..."

Regarding the phrase "especially the highly expressed individual subtypes within it", we acknowledge that this description is indeed insufficiently rigorous in the absence of specific experimental validation across multiple UGT

subtypes. Our current data focus exclusively on the single UGT subtype *BtUGT356B1*, without validation of other subtypes. Therefore, we have revised the sentence as follows (Lines 422-424): "...our research conclusion clearly indicates that *BtUGT356B1*, as a specific subtype of the UGT family with high expression levels, plays a crucial role in resistant whiteflies. "

13. *Line 376: "stimulating conditions". What does it mean? No stimulus has been described. These data are about RNAi experiment on a resistant strain.*

RESPONSE: Thank you for your meticulous review of the manuscript and your valuable suggestions. We acknowledge that the term was indeed ambiguous, as it failed to specify the exact type of stimulation, which could lead to reader confusion. We have therefore revised "stimulating conditions" to "under dsRNA treatment" to ensure terminological precision.

Revised Discussion (Lines 430-432): "We found that under treatment with dsRNA, activator or inhibitor, the total protein levels of ERK and P38 in whitefly remain stable, while their phosphorylation levels show significant dynamic changes. "

14. *Line 375-385: I find this clarification not satisfactory. Please try to modify as described in a comment above. This section should explain why after RNAi only phosphorylated proteins are affected. How that can influence expression of UGTs.*

RESPONSE: We sincerely thank you for your careful review of the manuscript and your valuable comments. In response to the above comment, we have made the following revisions (Lines 430-440):

"We found that under treatment with dsRNA, activator or inhibitor, the total protein levels of ERK and P38 in whitefly remain stable, while their phosphorylation levels show significant dynamic changes. This is because in a highly dynamic signaling system, ERK kinase activity and phosphorylation do not strictly depend on the total abundance of ERK. RNAi preferentially targets newly synthesized, signaling-competent ERK, thereby reducing the active fraction without substantially altering total protein levels, which is consistent with the characteristic of the MAPK pathway that achieves rapid signal transduction through post-translational modification, and the phosphorylation levels of ERK and P38 can be regulated independently of their total protein levels. "

Regarding the impact of such changes in phosphorylation status on UGT expression, the revised Discussion is as follows (Lines 451-460):

"The MAPK pathway, as a key node in the cellular signal transduction network, activates downstream substrates through cascade phosphorylation

reactions, with ERK and p38 kinases serving as core components. Their phosphorylation status is a prerequisite for activating downstream transcription factors. When the phosphorylation status of the MAPK pathway changes, it affects the activity of transcription factors regulating UGT expression via signal cascades, thereby modulating UGT expression. In resistant whiteflies, dynamic changes in ERK/P38 phosphorylation levels regulate the expression of *BtUGT356B1* through this mechanism, consequently mediating resistance to neonicotinoid insecticides. "

We sincerely appreciate your expert guidance, which is of great significance for refining our manuscript and deepening the research quality.

15. *Line 413: "exhibit a unified resistance mechanism" this claim can not be proved as only the role of UGTs has been investigated. Different strains, which also present different resistant level, might have evolved other mechanisms (as also described above in discussion). Needs to be rephrased.*

RESPONSE: We appreciate the reviewer's meticulous review. We have revised the relevant expressions to clarify that this study solely focuses on the metabolic resistance mechanism mediated by UGTs, while fully acknowledging that other resistance mechanisms may exist in whitefly populations with varying resistance levels. The revised content is as follows (Lines 497-504):

"This study clarifies that whitefly mediates metabolic resistance to neonicotinoid insecticides through the up-regulated expression of UGTs, and uncovers the mechanism by which the MAPK signaling pathway activates the transcription factor HR39, ultimately enhancing the detoxification capacity of UGT enzymes. This finding suggests that among the various resistance mechanisms emerging under the selection pressure of neonicotinoid insecticides, this pathway has the potential to be an important metabolic resistance mechanism."

16. *References to supplementary figure 1c, 2, and 3 are totally absent in the text.*

RESPONSE: We sincerely appreciate reviewers' meticulous corrections. We have added citation references to Supplementary Figure 1c, Figure 2 and Figure 3 in the corresponding positions of the main text, with specific revisions as follows:

Revised Results (Lines 150-153): Phylogenetic analysis indicates that *BtUGT356B1* from whitefly forms a clade with *Trialeurodes vaporariorum*, revealing a close evolutionary relationship between the two species (Supplementary Fig. 1C).

Revised Results (Lines 153-158): In addition, the 10 candidate UGT genes are

respectively distributed across chromosomes Chr01, Chr04, Chr05, and Chr10, with UGT genes on Chr10 (*BtUGT353B1*, *BtUGT353C1*, *BtUGT352A4*, and *BtUGT352A5*) showing a relatively concentrated distribution (Supplementary Fig. 2). These UGT genes form multiple clades and exhibit differences in the number of exons (Supplementary Fig. 3).

17. *Please, double check for typos, words in italics and be sure all supplementary material is cited in the appropriate way.*

RESPONSE: Thank you for your meticulous feedback. We have thoroughly reviewed and revised the manuscript according to your comments. The updated version has been carefully reorganized to ensure accuracy in content and compliance with formatting guidelines. We sincerely appreciate your valuable suggestions, which have significantly improved the quality of our manuscript.

Reviewer #3 (Remarks to the Author):

As an additional reviewer, I primarily focused on concerns previously raised by Reviewer 1 and whether these issues have been satisfactorily addressed in the revised manuscript. Overall, the manuscript has been substantially improved through the revision process, and most of the concerns raised by Reviewer 1 have been adequately addressed. The revisions have strengthened both the presentation and interpretation of the data. Nevertheless, a few points may still benefit from further clarification to improve the rigor of the study and the clarity of conclusions.

RESPONSE: We sincerely appreciate your dedicated efforts in verifying the adequacy of our responses to the prior concerns raised by Reviewer 1. Your professional judgment and targeted suggestions are of great value for further enhancing the rigor of our study and the persuasiveness of the conclusions. We will address every one of your concerns point by point, add necessary supplements to the corresponding positions in the manuscript, and mark all new changes with highlights. The exact page and line numbers of each modification will be clearly listed in our response letter for your easy verification.

1. Regarding Reviewer 1's comment #1, the revised text improved but remains somewhat general. The discussion could benefit from a more mechanistic description of how transcriptional regulation contributes to metabolic resistance. For example: "During the evolution of metabolic resistance, transcriptional regulatory mechanisms play a critical role in connecting insecticide exposure with resistance phenotypes. Insecticide-induced or constitutively activated transcription factors can upregulate detoxification genes, including cytochrome P450s, glutathione S-transferases, and carboxylesterases, thereby enhancing insecticide metabolism and detoxification. Increasing evidence suggests that such regulatory mechanisms are important contributors to the evolution of metabolic resistance in insect pests."

RESPONSE: We sincerely appreciate the valuable suggestions put forward by the reviewer. We fully agree that supplementing the mechanistic description between transcriptional regulation and metabolic resistance can further enhance the rigor of this manuscript. The mechanistic elaboration mentioned in your example has been fully supplemented to Lines 63-70 of the revised manuscript.

2. Regarding Reviewer 1's comment #5, the authors have adequately clarified the timing of gene expression measurements relative to LC50 determination, and the distinction between constitutive and inducible expression has been appropriately incorporated into the results section. However, some additional details would help readers to better evaluate the data. The author states that "only those individuals that remained alive and in good physiological condition

after insecticide treatment were selected for RNA extraction". It would therefore be useful to indicate how many individuals were excluded prior to RNA extraction, and whether all surviving insects were pooled or only a selected subset? Such information would help readers assess the potential impact of survivor bias. Given that 50mg/L is close to the LC50 of the S strain, selecting only the healthiest surviving individuals could potentially influence the observed expression patterns.

RESPONSE: We greatly appreciate the insightful and constructive comment, which helps us further clarify the experimental details and eliminate potential ambiguity in data interpretation.

We have now added the following supplementary content to the Materials and Methods section (Lines 623–630):

Three biological replicates were set up for each treatment, with approximately 60 adult whiteflies of standardized developmental stage initially introduced per replicate. After 3 h, 6 h and 12 h of exposure to the 50 mg/L insecticide treatment, a vitality assessment was performed on all surviving individuals. Specimens with abnormal physiological manifestations including sluggish movement and appendage tremor were excluded, to ensure no less than 30 healthy individuals with normal mobility and consistent physiological status were retained in each replicate for subsequent total RNA extraction.

3. In addition, the newly added discussion suggesting that "high-dose insecticides may directly damage physiological functions... inhibit stress response pathways..." is reasonable and plausible. However, as direct evidence for this is not provided in this study or by references., it maybe more appropriate to present this interpretation as a possible explanation rather than a definitive conclusion (e.g. "one possible explanation is that....").

RESPONSE: We sincerely appreciate the reviewer for this professional and thoughtful suggestion, which helps us avoid over-interpreting the results and make the discussion more rigorous and objective.

We have revised the corresponding statement in the discussion section (Lines 372-375): "One possible explanation is that high-dose insecticides may directly damage the physiological functions of whiteflies, inhibit their normal stress response pathways, and prevent the effective activation of gene expression regulatory mechanisms. "

4. A related point is that the alternative explanation raised by Reviewer 1 has not been directly explored experimentally. Specially, the lack of inducible expression in the S strain may reflect the treatment conditions rather than an intrinsic inability to induce expression. Because the same concentration (50mg/L) was applied to both S and R strains despite their substantially different

LC50 values, the physiological stress experienced by the two strains is unlikely to be equivalent, therefore, the possibilities that treatment conditions contributed to the observed differences should be acknowledged as a limitation in the discussion.

RESPONSE: We sincerely appreciate the reviewer for raising this highly valuable critical perspective. We fully agree that, given the significant difference in LC₅₀ values between the susceptible (S) and resistant (R) strains, the two strains are not subjected to equivalent physiological stress intensities when treated with the uniform 50 mg/L concentration. This variable may explain the absence of inducible gene expression observed in the susceptible strain, rather than being solely determined by the inherent difference in inducibility between the two strains.

We have supplemented the corresponding content in the Discussion section of the revised manuscript (Lines 375-383): "It should be noted that the S and R strains were treated with a uniform insecticide concentration of 50 mg/L. There is a significant difference in their LC₅₀ values, and the actual physiological stress intensities they experience are not equivalent. Therefore, the observed non-inducible expression phenotype in the S strain may be partially affected by the treatment conditions, which cannot be completely equated to the fact that the S strain itself lacks the activation capacity of this inducible pathway. This confounding factor should be fully taken into account when interpreting the difference in gene response between the two strains."

5. Regarding Reviewer 1's Comment #6, the authors have provided additional clarification regarding the apparent discrepancy between Fig 2B and 2F, however, some uncertainty remains. The explanation that the similarity observed in Fig 2B may reflect either the comparable basal expression levels or experimental error does not completely reconcile the difference between the two figures. If these data were generated from independent experimental batches, it would be helpful to provide additional information regarding normalization procedures, quantitative analysis, and any limitations associated with cross-batch comparisons.

RESPONSE: We greatly appreciate the reviewer for pointing out this detailed issue. We fully agree that supplementing relevant information can eliminate the uncertainty in interpreting the two figures and further enhance the credibility of the data.

1. Supplementary experimental background and normalization procedures for the two sets of data

We have explicitly annotated in the Materials and Methods section (Lines 675-677): The samples treated with time-gradient exposure and the samples from different strains belong to two completely independent experimental batches.

We have added the following content in the Materials and Methods section (Lines 677-680): For both independent experiments, electrophoresis, membrane transfer and antibody reagents were prepared in the same batch. β -actin was used as the internal reference to eliminate systematic deviations caused by reagents and operations across different batches.

Materials and Methods (Lines 637-641): *RPL29* and *EF-1 α* were used as the reference genes and the results were normalized via the $2^{-\Delta\Delta C_t}$ method⁶⁹. The relative expression level is calculated using the control group from the same batch as the benchmark. All qPCR reaction systems are kept consistent to avoid direct comparison of values across different batches.

2. Supplement and complete the quantitative analysis results

We have updated the supplemented quantitative statistical results to the corresponding results section (Lines 161-169): Under exposure to 50 mg/L TMX, the expression of the *BtUGT356B1* in R^{#1} strain shows an upward trend with the treatment time. After 6 h of treatment, the relative expression level was significantly increased by 1.9-fold ($P=0.0003$); after 12 h, the up-regulation increased to 2.5-fold ($P=9.07\times 10^{-6}$). The 50 mg/L IMI exposure targeting the R^{#2} strain showed that the *BtUGT356B1* also presented a time-dependent induction characteristic. After 6 h, the expression of *BtUGT356B1* was significantly up-regulated by 1.4-fold compared with the control group ($P=0.0037$); after 12 h, the up-regulation increased to 1.6-fold ($P=0.0002$).

3. Clarify the Boundaries and Limitations of Cross-Batch Comparison

We have added a supplementary statement in the Results section (Lines 188-192): It should be noted that Figure 2B focuses on the temporal dynamic changes of the samples, while Figure 2F focuses on the differences in basal expression among different strains. Their experimental treatment conditions and sample collection logic inherently do not have the premise for direct horizontal comparison.

6. Finally, the statement "RNAi preferentially targets newly synthesized, signaling-competent ERK" appears central to the author's interpretation of the ERK phosphorylation data. If available, it would be helpful for the authors to provide experimental evidence or appropriate literature reference supporting this mechanism.

RESPONSE: We greatly appreciate your valuable comments, which are of significant guiding importance for us to improve the mechanistic interpretation of our paper. We have supplemented two references in this field in the Discussion section of the revised manuscript to support the proposed mechanism from three aspects: direct evidence from the *B. tabaci* system, the inherent properties of the MAPK pathway, and the mechanism of RNAi action.

The detailed explanations are as follows:

First, the previously cited direct research evidence on the MAPK pathway in *B. tabaci* confirms that the GPCR-MAPK signaling axis can directly regulate the downstream trade-off effect between insecticide resistance and reproductive fitness through the phosphorylation cascade of p38 and ERK. Throughout the entire regulatory process, no statistically significant difference was observed in the total protein abundance of ERK and p38, which provides direct experimental support for the proposition that "phosphorylation levels are regulated independently of total protein levels" in insect systems.

[33] Fu, B. et al. GPCR-MAPK signaling pathways underpin fitness trade-offs in whitefly. *Proc Natl Acad Sci U S A.* **121**, e2402407121 (2024).

Second, we have cited a classic study on the regulatory mechanism of the MAPK pathway, clarifying that the core activation logic of this pathway relies on site-specific phosphorylation modifications. It can complete the signal transduction of the entire pathway within minutes after external stimulation without depending on the upregulation of total protein expression. This property is the inherent biological basis for the MAPK pathway to achieve rapid intracellular responses, which is consistent with the phenomenon of "stable total protein and dynamic changes in phosphorylation" observed in this study.

[55] Cargnello, M. & Roux, P.P. Activation and function of the MAPKs and their substrates, the MAPK-activated protein kinases. *Microbiol. Mol. Biol. Rev.* **75**, 50–83 (2011).

Third, we have supplemented a classic literature on the action mode of RNAi, which confirms that RNA interference preferentially targets and degrades nascent mRNA to block the production of newly synthesized active signaling proteins, and will not cause significant disturbance to the mature total proteins that have already existed stably. This explains the experimental result in this study that the total protein level did not change significantly after dsRNA treatment, while the active phosphorylated components decreased specifically.

[56] Niu, J., Chen, R., Wang, J.J. RNA interference in insects: the link between antiviral defense and pest control. *Insect Sci.* **31**(1), 2-12 (2024).

All the above newly added references have been fully integrated into the Discussion section of the revised manuscript.

We once again thank the reviewer for the constructive comments. This supplementation further consolidates the theoretical basis for the mechanistic interpretation of our paper, which greatly enhances the academic rigor of this study.