"Genome-wide Identification of Seven in absentia E3 ubiquitin ligase gene family and expression profiles in response to different hormones in *Uncaria rhynchophylla"*

Thank you for taking the time to review my paper word for word, and for providing valuable suggestions for revisions. According to your suggestions, we have made the following modifications.

1. Describe the full name of SINA.

Response: Thank you for your advise. We have supplemented it in our manuscript. The full name of SINA is Seven in absentia.

2. Lines 115- 117 is an incorrect statement. "The number of aa varied from 206 aa (UrSINA6) to 388aa (UrSINA1)…..MW, which ranged from between 23.63 kDa (UrSIA6)……., with an average of 37.46 kDa" is incorrect. According to Table 1, it varied from 206 aa to 506 aa (UrSINA7)…..MW, which ranged between 23.63 kDa (UrSINA6) and 57.46 kDa (UrSINA7), with an average of 40.55 kDa.

Response: Thank you for your revision. It was an error in our description of the results, and we have revised it in our manuscript based on your feedback.

3. Line 117-118. This is an incorrect statement. If a protein's pI value is higher than 7.0 (pI > 7), it is considered a basic protein. Conversely, if a protein's pI value is lower than 7.0 (pI < 7), it is considered an acidic protein.

Response: Thank you for your detailed modifications. We made mistakes in describing acidic and basic proteins, and we have corrected it according to your suggestion.

4. Line 119. The statement that only UrSINA7 is stable is not correct because both UrSINA4 and UrSINA7 have essentially the same value (38.0) of instability index. Explain the criteria distinguishing between the stability and instability of proteins based on the instability index.

Response: Thank you for your advise. The correct statement is "Among the ten UrSINA proteins, UrSINA2, UrSINA3, and UrSINA5 were basic proteins (pI > 7), and the remaining proteins were acidic protein (pI < 7). Besides this, except for UrSINA4 and UrSINA7, which is a stable protein (Instability index < 40), other SINA proteins are unstable proteins (Instability index > 40)."

5. Line 121- “UrSINA1/4 were only located in the nucleus.” To support this claim, description of Table 1 needs to be clarified using demarcation lines between the rows to identify which ones are located in nucleus and/or mitochondrion.

Response: Thank you for your advise. We have supplemented the demarcation lines in Table 1.

6. Line 149-150. The present tense “have” should be used instead of past tense “had.”

Response: We have corrected it in our manuscript.

7. Line 219. “UrSINA6 showing the lowest expression levels in materials.” This is an incorrect and unambiguous statement. According to Fig. 7, UrSIN4 exhibited the lowest expression in stem and leaf. All ten UrSINAs appear to be expressed at similar levels in root under normal conditions.

Response: Thank you for your rectification. We have rewritten this part. “Ten *UrSINA* genes are expressed in the roots, stems, and leaves, with *UrSINA9* showing the highest expression levels in stems and leaves, while *UrSINA4* showing the lowest expression levels in leaves. All ten UrSINA genes appear to be expressed at similar levels in root under normal condition. And the expression levels of UrSINA5/6/7 are indistinguiable between the roots and leaves. ”

8. Line 219-220 statement is entirely incorrect. UrSINA4 showed the lowest expression in leaf; expression levels of UrSINA5/6/7 are indistinguishable between the root and leaf. The P value indication of leaf UrSINA4 is missing despite the apparently significant reduction in its expression in leaf.

Response: Thank you for your rectification. We have added the significance in Figure 7, and we have rewritten the results in our manuscript.

9. Line 221-222 must be clarified. Provide the reference supporting this statement. Also, it is not clear what type of “most genes” show higher expression levels in other tissues. What do you mean by “other tissue”? If most genes refer to UrSINAs, then this statement does not make sense at all.

Response: We have deleted this statement in our manuscript based on your suggestion.

10. Line 233. Provide references supporting this statement.

Response: Thank you for your suggestion. We have added the reference supporting this statement in our manuscript.

11. In section 2.7. The qRT-PCR-based expression analysis of UrSINAs was implemented using root tissue. Authors must explain why root tissue was used instead of leaf or leaf when UrSINAs are relatively expressed at much lower levels in root compared to leaf and stem, as shown in Fig. 7, and the stem of *U. rhynchophylla* is the main site for medicinal compounds.

Response: Thank you for your questioning. First, the main site of *U. rhynchophylla* is stem, but the alkaloids in roots can still be detected in the roots, with the expression of key enzyme genes could be detected. Second, we calculated the relative expression level and used the expression level of genes in roots as a reference to represent the expression of genes in stems and leaves. Third, we have consulted some references and found that many exogenous ABA hormones have been applied in the roots, such as kiwifruit, apple, and *S. miltiorrhiza*. They were all collected roots as experimental materials, rather than fruits.

12. Fig.8b and Fig. 9b. In which tissue was used for analyzing expression patterns of the 12 key genes under ABA and MeJA treatment?
Thank you for your suggestion. We have supplemented in Figure 8b and Figure 9b.

13. UrSINA4/7/10, which do not have ABA-responsive cis elements (Fig. 6), exhibited no differential temporal expression patterns (Fig. 8). In contrast, UrSINA2/6/9 showed differential temporal expression patterns, and UrSINA9 was first up-regulated at 1 h after ABA treatment despite the absence of ABA responsive cis-acting elements. Explain this apparent discrepancy.

Response: Thank you for your questioning and rectification. We think that the analysis of *cis*-acting elements is based on predicted results, while qRT-PCR analysis is a validation experiment. We have stated in our manuscript "The qRT-PCR results show that the expression levels of *UrSINA3*, *UrSINA4*, *UrSINA7* and *UrSINA10* exhibited no differential temporal expression patterns under ABA treatment, implying that they did not respond to the ABA hormone. Among them, *UrSINA4*, *UrSINA7* and *UrSINA10* which do not have ABA-responsive cis-acting elements (Figure 6). *UrSINA1*, *UrSINA2*, *UrSINA6*, and UrSINA9 showed differential temporal expression patterns. and *UrSINA9* was first up-regulated at 1h and had the highest expression levels at 12 h after ABA treatment, indicating that *UrSINA9* is the most sensitive gene in response to ABA hormone (Figure 8). The same situation also occurs in kiwifruit and *Salvia miltiorrhiza*, for exmaple, AcSINA4/8/10/11 did not contain ABRE *cis*-acting element in promoter region, but they were all induced by ABA; There was not found any ABA responsive elements in SmCUL2, while it was responsed to ABA hormones."

14,15. Line 241-244. This is an incorrect statement. According to Fig. 8a, not UrSINA5/6 but UrSINA9 was first up-regulated at 1 h and the highest expression levels at 12h after ABA treatment. UrSINA9 is the most sensitive gene in response to

ABA; Line 253-254. This is an incorrect statement. UrSINA1 showed significantly positive correlation with Ur7DLH and UrSTR, but not with UrLAMT.

Response: Thank you for your suggestions. We have corrected the mistakes in our manuscript.

16, 17. Line 262-263. Authors ignored the following important point: Unlike the other UrSINAs, UrSINA4 showed a relatively stable (less variable) temporal expression pattern, and this pattern is consistent with the observation that only UrSINA4 lacks MeJA-responsive cis-acting element; Description of reference 4 (Line 503) is incomplete, missing in the name, volume and page number of literature.

Response: Thank you for your suggestion. We have rewritten and supplemented the relevant content in manuscript. Thank you!

18, 19. Line 309-311. Rewrite this inept and redundant sentence by simply stating – The number of UrSINA paralogous genes indicates a diversification of UrSINA functions in both spatial and temporal expression, as well as substrate specificity; Line 313-315 needs to be clarified: "According to the predicted……. different functions (Table 1)." Single or multiple UrSINA proteins might have different functions? The predicted cellular location of UrSINA suggests that it is involved in regulating the stability of nuclear and mitochondrial proteins.

Response: Thank you for your suggestion. We have made adjustments in the manuscript.

20. Line 383. Provide the reason why the SINA ubiquitin E3 ligase targets crucial enzyme genes of TIA pathway rather than transcription factors controlling the expression of genes involved TIA biosynthesis pathway.

Response: The results of co expression association analysis are speculative, and whether UrSINA can participate in the synthesis of TIAs requires subsequent functional validation. We wrote it in the manuscript as follows: "Previous study have shown that ABA could increased the content of TIAs by activating the expression of genes in TIAs biosynthesis pathway in *C. roses*. We found that ABA can accumulate the content of rhynchophylline and isorhynchophylline (data is unpublished). Most key enzyme genes are up-regulated under ABA treatment (Figure 8b). And according to co-expression, *UrSINA*1 was significantly positively correlated with Ur7DLH and *UrSTR*, and *UrSINA10* showed a negative corelation with *UrSLS* (Figure 8c). This result shows that the UrSINA gene and key enzyme genes could share similar regulatory effects as a result of the ABA treatments, implying that these UrSINA genes might participate in TIAs biosynthesis in *U. rhynchophylla*."

21. Line 353-355 statement is essentially the same as line 389-392. This redundancy must be removed.

Response: Thank you for your suggestion. We have corrected in manuscript.

22. Discussion is mostly repetition of the results. What are the potential substrates of the identified 10 UrSINAs? Authors needs to discuss this point. This could be inferred from the same clade group of phylogenetic tree and/or amino acid sequence similarities in SINA domain (responsible for the interaction with target proteins) between the UrSINAs and other species' SINAs and the known substrates? For examples, SINAT5 (SINA of Arabidopsis thaliana 5) ubiquitinate the transcription factor NAC1. ATG6 is a target of the SINATs in Arabidopsis. OsDIS1 (Oryza sativa drought-induced SINA protein is a negative regulator of drought response and directly interacts with and promotes the degradation of OsNek6. Conversely, SINA2 positively regulates drought tolerance in an ABA-dependent manner in Arabidopsis. A banana SINA E3 ligase MaSINA1 was recently found to ubiquinate and promote the degradation of MaICE1, which positively regulates cold tolerance i. These are a few examples, and there may be many others.

23. Discuss the significance of the presence of light-responsive cis-acting element in UrSINAs. It has been reported that SINAT protein levels are decreased in the dark and increased in the light, suggesting that darkness may promote autodegradation of SINATs.

Response: We found that UrSINA7 was clustered with OsSINA4 and OsSINA6, the study has shown that OsSINA4 (OsDIS1) acted as a negative regulator in drought response, so we think UrSINA7 may have similar function. And previous study have shown that the expression of resistant genes were regulated by MYB transcription factor. For instance, PbrMYB21 was able to positively contribute to drought tolerance by binding to the MYB recognition sites in the PbrADC promoters region [53]. Many MYB binding sites were identified in promoter region of UrSINA genes (Figure 6), therefore UrSINA genes might likely be regulated by the MYB transcription factors, which involved in mediating drought stress signaling.

Response: Thank you for your suggestion. We have read this manuscript, and supplemented as follows: "Interestingly, it has been demonstrated that the degradation of SINATs is self-regulated in A. thaliana, and SINATs were degraded as a result of thier interaction with the photoreceptors cryptochrome (CRY1) and phytochrome B (phy B) in the cytoplasm under dark condition. Moreover, phyB and CRY1 were dissociated from SINATs by red and blue light induced and was the primary factor influencing the SINATs accumulation."

24, 25,26. Line 397 is an incorrect statement. SINA gene family was not identified from whole genome transcriptomic data and qRT-PCR expression analyses but by

in-silico protein domain homology searches; Line 436 is an incorrect statement. SINAs are not transcription factors but E3 ubiquitin ligase; Line 400 is an inept sentence – “bioinformatics were analyzed” Bioinformatics is an analysis tool.

Response: Thank you for your rectification. We have made modifications in the manuscript to address these three issues.

Finally, thank you again for your valuable feedback and understanding! If you have any better suggestions, please let me know.