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Peer Review File

A naturally occurring SNP modulates thermotolerance divergence among grapevines

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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)
In this manuscript, Chen et al. found that TTC4, a novel gene encoding a WRKY transcription factor, positively regulates thermotolerance in grapevine. TTC4 directly upregulatesthe expression of HSP18.1 and APX3, both of which are heat-stress responsive genes and enhance thermotolerance in grapevine. Importantly, SPL13, a transcription repressor, binds to the GTAC element on the TTC4 promoter to repress TTC4 expression in heat-sensitive cultivars, and cannot recognize the GTAT element on the TTC4 promoter in heat-tolerant wild species. Although thismanuscript has sufficient evidence and novelty, several problems still need to be resolved.
Comments for the MS:
1. Fig.2a-f, the authors showed the phenotype and related data of OE-TTC4 and Si-TTC4 to prove that TTC4 confers thermotolerance in grapevine. This is the essential result of themanuscript. The transient expression system was utilizedfor OE and RNAi. How to achieve stable expression of TTC4 in different plantlets of three biological replicates?
2. Extended Data Fig. 4b, it is not clear what the PCR result represents? DNA of transgenic TTC4 or the expression level of TTC4 inoverexpressing Arabidopsis lines. It is preferably to provide RT-qPCR data to detect the expression level of TTC4. In addition,the sentence “M indicates the marker; P indicates the plasmid of OE-TTC4.” should be deleted.
3. Fig. 6e-g,SPL13wasoverexpressedinY2 grapeleaves (OE-SPL13) and reduced the thermotolerance of Y2 grape, of whichGTAT(-417) in TTC4 promoter cannot be bound by SPL13. “the expression of TTC4T(-417)remained relatively unchanged in grapevines transiently overexpressing SPL13 (Extended Data Fig. 9b).” The results indicate the phenotype of reducing thermotolerance in OE-SPL13Y2 grape is not dependent on TTC4 expression. It seems inconsistent with the conclusion supported in this MS. However, the phenotype of overexpressing SPL13 (OE-SPL13) in JW grape leaves, which contains GTAC (-417) in TTC4 promoter, should be provided here.

Reviewer #2

(Remarks to the Author)
Grapevine is an important perennial fruit crop. Although it is well-adapted to adverse environmental conditions, it is quite sensitive to extreme temperatures. Due to climate change, global warming in particular, many traditional viticultural areas may not be appropriate for winegrowing in the near future, unless we identify/develop heat-tolerant genotypes. However, despite advancements, our understanding on the molecular mechanisms providing thermotolerance in grapevines remains largely elusive.
In this study, the authors unveil compelling findings that shed new light on the role of a WRKY transcription factor (referred to as TTC4) in enhancing grapevine thermotolerance through upregulating HSP18.1 and APX3. They provide convincing data showing that sequence variation (C/T) at a SNP in the TTC4 promoter (TTC4C/T(-417)) significantly affects the expression of TTC4. They also show that SPL13, a SQUAMOSA promoter-binding protein-like (SPL) gene, whose expression decreases under high temperature, may bind to TTC4C(-417), but not to TTC4T(-417), and repress its expression. Interestingly, this particular C/T SNP variation is associated with diverse thermotolerance in grapevines: Vitis vinifera cvs. tends to have SNP-C (-417), whereas wild vines likely have TTC4T(-417) genotypes, thus being more heat-tolerant. These findings are every important considering that this particular SNP could serve as a marker for rapid screening thermotolerant vine germplasm or as a QTL for breeding.

However, there are some points that need the attention of the authors:

1. The literature and databases present VIT_204s0023g00470 (TTC4) as the gene LOC100253049 (protein XP_059592356). It was previously described by Wang et al. (2014, PMID: 26504535) as a member of Group I of the WRKY gene family, as shown here. The authors gave it the name VvWEKY13 and it appears with this name thereafter (e.g., https://www.sciencedirect.com/science/article/pii/S0925521418308196). They also present data showing that the gene is highly expressed in stamen and pollen tissues of grape, being closely related to Arabidopsis AtWRKY34, which has a role in coordinating gene expression during the formation of the tapetum and microspores. The present article does not demonstrate any of the aforementioned aspects. May this gene have functions other than the thermotolerance presented here? In addition, is it proper to change the name of this gene?

2. The definition of VvWEKY13 protein in NCBI (NCBI Reference Sequence: XP_059592356.1) is “WRKY transcription factor SUSIBA2 isoform X3 [Vitis vinifera]”. Blastp searches reveal high homologies of VvWEKY13 with WRKY transcription factor SUSIBA2 from various plant species. How does this correlate with the present results?

3. Monitoring changes in Fv/Fm can provide insights into the impact of heat stress on plant health and thermotolerance. However, Fv/Fm values may not be the sole indicator of thermotolerance. Electrolyte leakage was also applied in some cases to verify thermotolerance, but not in all cases/experiments. Is there any explanation?

4. Ln. 120-121. In Suppl. Fig. 1b, it appears that there is a notable signal on Chr4, whereas the presence of a significant signal on Chr7 is not obvious.

5. Lns. 305-328. An in-depth critical comparison with the present results would be more appropriate. The same for Lns. 330-356.

6. Lns. 486-487. I think the term “haplotype” may not correct here, since there is only one locus (SNP) under consideration. Consider "genotype” instead.

7. I also noticed few minor English errors or typos.

Fig.1g. The phylogenetic tree needs interpretation. How the “similar WEKY proteins” were selected. Any conclusion? Also, the names of the proteins are not informative. Maybe NCBI accession numbers would help.

Fig. 2h-i. Data for TTC4 conferring thermotolerance to Arabidopsis are not convincing. In Arabidopsis, only phenotypic data and the survival rates are presented. Data from the relative electrolyte leakage, Fv/Fm values, and control (empty vector) are missing. I wonder if Hsp18.1 and APX3 genes were also upregulated? Finally, how likely it is for TTC4 to share the same function in Arabidopsis?

Reviewer #3

(Remarks to the Author)
The paper describes the identification of a gene involved in the control of thermotolerance in grapevines. The authors also identify a putative single nucleotide, fixed for different alleles in Vitis vinifera and in all other wild species tested, that would be responsible for differences in expression that result in differences in thermotolerance. While I appreciated a lot all the functional validation experiments that the authors carried out, I have some serious reservations on their premises, i.e. the genetic analyses and the identification of the putative candidate gene.
The main problem lies in the structure of the identified TTC4 gene. There is no indication in the paper either in the results or in the methods section or in the reporting summary on which reference sequence was used or on which gene prediction and annotation version was used. The gene identifier listed in the paper (VIT_204s0023g00470) would seem to indicate that they used the Vitis vinifera 12Xv0 genome assembly of the strain PN40024 (GCA_000003745.2)and that the gene models refer to the V2.1 gene annotation. The structure of the VIT_204s0023g00470 gene (for which I report hereunder both the predicted protein and the nucleotide sequence of the predicted mRNA) is however substantially different from the one reported here that instead seems to correspond to that of the previous version of the gene prediction and annotation V1.0 where the gene was labelled as VIT_04s0023g00470.
In the VIT_04s0023g00470 the predicted protein encoded by the gene is 502 aa long as reported in the paper and the ATG starting codon is located on chr4 at base 16666517 on the – strand. 417 bp upstream of it, at base 16666934, the reference sequence of PN40024 contains a C on the – strand that is the last base of a GTAC motif starting at base 16666937. The base at 16666934 is variable among Vitis species as a consequence of a C-T transition. All this matches what is reported in the paper even though there is no reference to any coordinate system. Based on this information the C-T polymorphism would fall in the putative promoter region of the gene as reported in the paper.
The VIT_204s0023g00470 gene prediction is however different, because it starts with the ATG starting codon much further upstream (on the – strand) at base 16674564, if we look 417 bp upstream we do not find either the expected GTAC motif or the reported G base , so all this makes us conclude that the whole paper was based on the previous gene prediction and annotation version. The VIT_204s0023g00470 prediction produces a predicted protein that is substantially longer that 512 aa with an additional 198 aa at the N-terminus of the protein and most importantly with 2 additional exons and a transcription starting site that is around base 16674777. This is fully supported by the following evidences:
1) the alignment of RNAseq reads that show that the correct gene prediction is not VIT_04s0023g00470 but rather VIT_204s0023g0047;

2) the new prediction performed on the new T2T assembly of PN40024 visible at NCBI (https://www.ncbi.nlm.nih.gov/nuccore/NC_081808?report=graph&v=18127871:18145836; https://www.ncbi.nlm.nih.gov/protein/XP_059592355.1?report=genbank&log$=protalign&blast_rank=2&RID=YGYAMSM1013) that match VIT_204s0023g0047 and not VIT_04s0023g0047
3) the location of the peaks for the histone modifications that are typical of TSS (H3K4me3 and H3K27ac) that are located in a region between 16674000 and 16675000 bp on chr 4 (Schwope et al. Plant Journal 2021).
4) Finally and perhaps most importantly the structure of WRKY genes in other species that corresponds to that of the VIT_204s0023g00470 prediction and not of the VIT_04s0023g00470 used here.
The implications of these differences in gene structure are of paramount importance for the paper under review:
1) the functional experiments involving the TTC4 gene used a truncated version of the protein, missing 198 aa at the N terminus
2) the identified SNP does not lie in the promoter but rather in the second intron of the predicted gene
3) the promoter that was cloned and used in the paper for functional experiments (that based on the primers provided in Supplementary Table 7 spans a region between bp 1666518 and 16668829) does not correspond to the promoter of the gene but rather to a small region of coding sequence and a large region of the second intron that includes approximately 1.3 kb annotated as a LINE transposable element (that in the grapevine genome are almost exclusively found within introns).

So I think it would be extremely important that the authors justify their findings and observation in light of these differences between their evidences and the evidences provided by others on the gene structure and possibly repeat their experiments using the correct protein sequence and promoter sequence. It is not unheard of that regulatory elements of different types (silencers, enhancers, insulators) are found in the introns but this would still imply that the functional experiments performed should be redone entirely in my mind.
I have some additional comments on the other genetic analyses:
1) the structure of the population used for GWAS is extremely unusual, being made of different species and interspecific hybrids as well as intraspecific genotypes. The reasons for choosing such a population should be explained
2) There is no reference to the genome assembly or gene prediction versions used
3) There is no detail on how the filtering of the SNPs used for the GWAS analysis was carried out. It is surprising that after whole genome resequencing of 121 genotypes belonging to 8 different species, a set of only 2M SNPs to be used for GWAS is identified
4) There is no estimate provided on what fraction of the phenotypic variance for the Fv/Fm is explained by the identified locus on chr 4. This is important to understand if the locus should be considered a QTL or rather a mendelian locus.
5) The interval identified by GWAS comprises 200 predicted genes. There is no explanation as to how they went from these 200 genes to the 84 considered for expression differences, nor how they went from these 84 to the 6 reported in Fig. 1d. There are other genes in the set of 84 reported that seem to be more upregulated under high temperatures that the 6 identified and VIT_204s0023g00470 does not seem to be the most induced in JX.
6) It is not clear how the GWAS on the presumed promoter of TTC4 reported at lines 226-229 differs from the initial one
7) At lines 279-282, the diversity within species (Vitis vinifera) cannot be compared with divergence between species (diversity in wild species) so that this difference in diversity cannot be used as evidence of selection acting in vinifera
I would have multiple other comments to make on other aspects of the paper but I think that prior to addressing those the authors should address the issue of the predicted gene structure.

Predicted protein in V2.1. The start of the predicted protein used in the paper corresponds to the MSQHKEP motif found after 198 aa in this protein
>VIT_204s0023g00470.1 gene=VIT_204s0023g00470
MDGMDSTRINSGSIAERRAAKFGFDASIIKTPRFRCSRLLALPAARSPPLIIPPGISPTVLLDSPIMLPN
TQAQLSPTTGTFQVPSLIHEGSVNSVAPTVDGDQANNFSASGKFKSHANPISLPCFSSIEIQVSSPSDLA
QSFGAEVHYQTCAPTHSPVGFEFATEFSTEASAKNYVFDSATDVKVSNTMISDIPSDHMSQHKEPIHSEN
VGMHHIPEEQKGTYPSMGMGRTSEDGYNWRKYGQKSMKGSEHTRSYYKCTHLDCPMRKKVQQSHDGQITE
IIYKGGHNHPKPLPSRRSALGSTLPFNEMSGLGEGGGSSVRVEGGSIWRNVQPGSKNDRAGSDWRANGLE
RTSSTSAVSALSNSLSNTGGISMGIFESAGTPDLSLTVASQDDGEDGATQGSISLGDDADDEGSQSKKRT
VREPRVVVQVECESDVLNDGYRWRKYGQKVVKGNLHPRNYYKCTSTGCSVRRHVERASNNQKSIIATYEG
KHNHEVPAARNSSHVNSSGGNLPSAAPGAQSALALHRNANAPRPEALLQDLVPHFDIKPEFSNQYIRHSM
LGNFANDMKFGPSSLYSMQFPPLQNTMLYAPFGLDSNNADPHQPGSVAPVAPDFPISLPLNLPPPANLGL
PGFDFNSHGNPIGQVEPYFVGQQLQENDMRFLHPKEEKKDDIGDATTSCSSSTIYH

Predicted mRNA in V2.1
>VIT_204s0023g00470.1 gene=VIT_204s0023g00470 CDS=214-2274
CTCCCTTCTTTAGGCTTTAAATTAAAATCCACACTCCCAACCTTCCCGGAGAAAGAAAATTGTGATGGTA
GGAAACAACGTCAAAGGACGTTGTTTCCCACCAATCTATCAGTTTCAGCTCCACCCTCGAAACGTAAGCT
TCAGAAATCTGGGGTTCTACCCAGTATGGCTGCTGCTGCTACCTCCATGGAAGTCTGAGTCATTTTTTCC
TCCATGGATGGCATGGATTCGACTAGAATAAATAGCGGGAGCATTGCGGAGAGGAGAGCCGCCAAGTTTG
GCTTTGATGCTTCCATAATCAAAACGCCGCGTTTCAGGTGTTCCAGACTCTTGGCGTTGCCGGCGGCTCG
GTCGCCGCCCCTCATTATACCTCCGGGCATCAGTCCCACCGTCTTGCTCGATTCTCCGATCATGCTCCCC
AACACTCAGGCACAACTGTCTCCTACAACTGGAACTTTTCAAGTGCCTTCTCTCATTCATGAAGGTTCAG
TAAATTCAGTGGCTCCTACTGTAGATGGTGATCAGGCCAATAACTTCAGTGCCTCCGGCAAGTTCAAATC
ACATGCAAATCCCATCTCTTTGCCATGCTTTTCTAGTATAGAAATTCAGGTTTCTTCCCCTTCCGATCTT
GCTCAAAGTTTTGGGGCTGAAGTTCACTATCAAACTTGTGCTCCAACACATTCACCAGTGGGTTTCGAAT
TTGCCACAGAATTCTCTACAGAAGCTTCTGCTAAAAACTATGTGTTTGATTCTGCCACTGATGTAAAAGT

TTCAAACACTATGATTTCTGATATACCTAGTGATCACATGTCTCAGCACAAGGAGCCTATCCACAGTGAG
AATGTAGGGATGCATCACATTCCAGAAGAACAGAAAGGCACATACCCTTCCATGGGAATGGGAAGAACTT
CAGAAGATGGATACAATTGGAGAAAATATGGGCAAAAAAGTATGAAAGGTAGTGAACATACAAGGAGCTA
TTATAAATGTACTCATCTAGATTGTCCAATGAGAAAAAAGGTACAACAATCACATGATGGTCAAATAACA
GAAATTATCTATAAGGGTGGTCATAACCACCCAAAACCACTGCCTAGTCGTCGATCAGCTCTTGGATCCA
CACTTCCATTTAATGAGATGTCAGGTTTGGGGGAAGGTGGTGGATCTAGTGTCAGAGTTGAAGGTGGCTC
AATTTGGAGAAACGTTCAACCAGGATCTAAGAATGATAGAGCTGGTTCTGATTGGAGGGCCAATGGTTTG
GAGAGGACATCCTCAACATCTGCTGTAAGTGCGCTCTCCAACTCCTTATCAAACACAGGAGGAATATCCA
TGGGTATATTTGAATCAGCAGGAACCCCAGATCTTTCCTTAACAGTTGCTAGTCAAGATGATGGTGAAGA
TGGGGCTACTCAAGGAAGCATATCACTTGGAGATGATGCTGATGATGAAGGTTCTCAGTCAAAAAAAAGG
ACTGTACGTGAACCAAGGGTTGTTGTCCAAGTAGAGTGTGAATCGGACGTCCTTAATGATGGATATCGCT
GGCGAAAGTATGGACAAAAAGTCGTCAAAGGCAATCTGCATCCTAGGAATTACTACAAGTGCACAAGCAC
TGGATGCTCAGTGAGGAGGCATGTGGAAAGAGCTTCCAACAATCAGAAATCCATAATTGCGACATATGAA
GGAAAACACAATCATGAAGTGCCTGCAGCCAGAAACAGCAGTCATGTGAATTCAAGTGGTGGAAATTTGC
CTTCAGCTGCTCCTGGTGCTCAATCAGCACTGGCTTTGCACAGGAATGCTAATGCCCCAAGGCCAGAAGC
ACTACTTCAAGATCTTGTCCCCCACTTTGACATAAAACCTGAATTCAGCAATCAGTATATAAGGCACAGT
ATGCTTGGGAACTTTGCTAATGATATGAAATTTGGTCCTTCCTCCCTTTATTCAATGCAGTTCCCTCCCC
TGCAAAATACCATGCTTTATGCTCCTTTTGGACTGGATTCTAATAATGCTGATCCCCACCAACCTGGTTC
AGTTGCTCCAGTAGCCCCAGATTTCCCAATTTCATTGCCATTGAATCTCCCCCCACCTGCAAATCTGGGT
CTGCCTGGCTTTGACTTCAACAGTCATGGAAACCCGATTGGTCAAGTTGAGCCATATTTCGTGGGGCAGC
AGCTTCAGGAGAACGATATGAGGTTCCTCCATCCTAAAGAGGAGAAGAAGGATGACATTGGTGATGCAAC
TACATCATGTTCATCATCAACAATATATCACTAGATCATGGGAAATTTTCCAACTTAAAGTTCTTTTCTT
TTGCATCTAAGTCATAATGGGAGCAAACTCTGCTACTCCTCTGCAATTTGGTTCTTTTATGATCAATTGG
TATCTTTTTCTCTTCTTTTCCCTTGTTTGCTTGTTCTTTTCAAATTTAAAGCATTTTAAGAATTTCCCAC
ACACACTGCACACTGGCATCTGTAACAAACTAGACTTTCCTAGTTCTCACATGTATCAGATAGTTCTTTC
TTTCATAGACATTGGTAGCTTAAAATGGTAGCAGAGCTCTTGTTGGTCAACTAAAGATTTCATGGCTGAT
GTATTGTAGGCTCATTATCCAAATAGCTCAAGCTTTTATGCAAGTTGGTAGCTTAACAAACTCTAATTAG
CATTTGATTCTGTTTTAGAGGTATGAATGCTGCTGAACTCTGAGTTTTCTGATTTTGAATGTCTGAAACT
ACTAATATCTTCTGTGATGCTCTCTACCGCTCTGCAGGGTTGCCCTTCTGTGTTGGTCTTCAACCATGAG
CAGCTTGCATTCTTATAGTAGCAGAATGGCAAGAAAAAAGGTAATGTGTCTTGACCCAAACCTTGGAGGT
TTTGAGGAAGTTGGTTACAGTCACTCCCACAAAATTTGTCATTAATTTTTTGTGGTGTGTGGGGCTTAGA
ACCCCTCTAAGTCAGCCTACTTGAGATTTCAACAAGGTGCAAAAAGTTTTTGTGATGTGCAGCGCTTAGG
ATCCGTCTCCTGTGTTGGCCTTCAACCATGAGCAGCTTGCATTCTTATAGTTGTAGAATGGCAAGAAAAG
AGTGCTTAGGATCCCTCTCTTCTGTGTTGGCCTTCAACCATGAGCAGCTTGCATTCTTATAGTTGCAGAA
TGGCAAGAAAAAAGGTGATGTATCTCAACCCCAAACCGTAGGTTTTGAGAAGTTGGTTATTGTCACTTCC
ACAAAATTTGTCATTAATTTTTTGTGATGTGAGGTGCTTAGGACCCTTCTAAGTCAGCCTACTCTAGACT
TCAACCATGAGCAGCTTGCATTCTGATAGTTGCAGAATGGAAAGAAAAAAGGACCCTCTAAGTCAGTCTA
CTCGAGAGTTCAACAAGTTGCAAAAATTTCTGTGATATGCGGTGCTTAAGACCCCTCTAAGCCAGCCTAC
TCGAGATTTCAACGAGGTGCAAGCAGTATCCAGAATGAGAACCAGCTTAAGAATGGAGGAGTGCATTTTC
AGTTCTATTGAGTTTTTTTTAGCTGCATTCATGTTGGAGATTTTGAACTCATATTCACTACTCTTGGTAT
CATAAATTATATTTATATTCTTCTTTCTTATACATTCAAGTCAGTAAAAGAAATGAGGTGCTAAATTTGG
GTCGATTTGTGTCGGGTTCGTGTCCTATTAAGAAAAA

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The revised manuscript has addressed previous comments. No new comments for changes on the revised manuscript.

Reviewer #2

(Remarks to the Author)
In my opinion, the authors have thoroughly addressed the comments and concerns raised by me and the other reviewers. So, I recommend publication in its revised form.

Response

Reviewer #1 (Remarks to the Author):

In this manuscript, Chen et al. found that TTC4, a novel gene encoding a WRKY transcription factor, positively regulates thermotolerance in grapevine. TTC4 directly upregulatesthe expression of HSP18.1 and APX3, both of which are heat-stress responsive genes and enhance thermotolerance in grapevine. Importantly, SPL13, a transcription repressor, binds to the GTAC element on the TTC4 promoter to repress TTC4 expression in heat-sensitive cultivars, and cannot recognize the GTAT element on the TTC4 promoter in heat-tolerant wild species. Although this manuscript has sufficient evidence and novelty, several problems still need to be resolved.

Comments for the MS:

1. Fig.2a-f, the authors showed the phenotype and related data of OE-TTC4 and Si-TTC4 to prove that TTC4 confers thermotolerance in grapevine. This is the essential result of the manuscript. The transient expression system was utilized for OE and RNAi. How to achieve stable expression of TTC4 in different plantlets of three biological replicates?

Response: Thank you for your question. To achieve reproducible overexpression (OE) and RNA interference (RNAi) in transiently transformed grape plantlets, we used the following methods: (1) Transient Overexpression: Six-week-old plantlets were vacuum-infiltrated (-90 kPa for 20 min) with *Agrobacterium* carrying the overexpression construct. After infiltration, the plantlets were rinsed with ddH_2O and blotted dry. They were then placed in ½MS medium and grown in a greenhouse at 25°C with a 14-hour photoperiod. (2) RNAi: One-year-old grape plants that had been germinated for 8 weeks were similarly vacuum-infiltrated (-90 kPa for 20 min) with Agrobacterium carrying the RNAi construct. They were rinsed with ddH_2O, blotted dry, and grown in small pots containing nutrient soil in a growth room at 25°C with a 14-hour photoperiod.

In each case, we assessed *TTC4* expression levels to confirm effective overexpression or RNAi. Our results showed significantly higher *TTC4* expression in OE plantlets compared to empty vector (EV) controls, and notably reduced expression

in RNAi plantlets compared to EV. By following this transient transformation approach (Kerschen et al., 2004; Jelly et al., 2014), we achieved stable overexpression of *TTC4* across multiple plantlets in three biological replicates. This system has also been validated for functional studies of *MYB30*, *HSFA2*, and *HSFB1* in our previous work (Mu et al., 2022; Liu et al., 2023; Chen et al., 2023). We have included these details in the revised manuscript. We hope this clarifies how we ensured reproducible transient expression in different plantlets and biological replicates.

Chen HY et al. The class B Heat Shock Factor HSFB1 regulates heat tolerance in grapevine. Horticulture Research. 2023, 10: uhad001.

Liu XN et al. Natural variations of HSFA2 enhance thermotolerance in grapevine. Horticulture Research. 2023, 10: uhac250.

Mu HY et al. MYB30 and MYB14 form a repressor-activator module with WRKY8 that controls stilbene biosynthesis in grapevine. The Plant Cell. 2023, 35: 552-573.

Kerschen A et al. Effectiveness of RNA interference in transgenic plants. FEBS Letter. 2004, 566: 223-228.

Jelly NS et al. Transient expression assays in grapevine: a step towards genetic improvement. Plant Biotechnology Journal. 2014, 2: 1231-1245.

2. Extended Data Fig. 4b, it is not clear what the PCR result represents? DNA of transgenic TTC4 or the expression level of TTC4 in overexpressing Arabidopsis lines. It is preferably to provide RT-qPCR data to detect the expression level of TTC4. In addition, the sentence "M indicates the marker; P indicates the plasmid of OE-TTC4." should be deleted.

Response: Thank you for your suggestions. We have provided the RT-qPCR result of *TTC4* in overexpression 41B suspension cells (Extended Data Fig. 5a). The sentence "M indicates the marker; P indicates the plasmid of OE-TTC4." has been deleted.

3. Fig. 6e-g, SPL13 was overexpressed in Y2 grape leaves (OE-SPL13) and reduced the thermotolerance of Y2 grape, of which GTAT(-417) in TTC4 promoter cannot be bound by SPL13. "the expression of TTC4T(-417) remained relatively unchanged in grapevines transiently overexpressing SPL13 (Extended Data Fig. 9b)." The results indicate the phenotype of reducing thermotolerance in OE-SPL13 Y2 grape is not

dependent on TTC4 expression. It seems inconsistent with the conclusion supported in this MS. However, the phenotype of overexpressing SPL13 (OE-SPL13) in JW grape leaves, which contains GTAC (-417) in TTC4 promoter, should be provided here.

Response: We appreciate your constructive comment and share your viewpoint. In the revised manuscript, we have included the phenotype of JX grape leaves overexpressing *SPL13* (OE-*SPL13*) (Fig. 6e-6g). The SNP (-417) in the *TTC4* promoter, mentioned in the original manuscript, has now been updated to SNP (7631) in *TTC4* intron 2-2 in the revision, reflecting the updated genome annotation. As anticipated, $TTC4^{C(7631)}$ expression was significantly down-regulated in grape plantlets overexpressing *SPL13* (Extended Data Fig. 12b). These results confirm that *SPL13* acts as a negative regulator of *TTC4* when the GTAC motif is present, thereby providing the phenotype data requested by the reviewer.

Reviewer #2 (Remarks to the Author):

Grapevine is an important perennial fruit crop. Although it is well-adapted to adverse environmental conditions, it is quite sensitive to extreme temperatures. Due to climate change, global warming in particular, many traditional viticultural areas may not be appropriate for winegrowing in the near future, unless we identify/develop heat-tolerant genotypes. However, despite advancements, our understanding on the molecular mechanisms providing thermotolerance in grapevines remains largely elusive. In this study, the authors unveil compelling findings that shed new light on the role of a WRKY transcription factor (referred to as TTC4) in enhancing grapevine thermotolerance through upregulating HSP18.1 and APX3. They provide convincing data showing that sequence variation (C/T) at a SNP in the TTC4 promoter (TTC4C/T(-417)) significantly affects the expression of TTC4. They also show that SPL13, a SQUAMOSA promoter-binding protein-like (SPL) gene, whose expression decreases under high temperature, may bind to TTC4C(-417), but not to TTC4T(-417), and repress its expression. Interestingly, this particular C/T SNP variation is associated with diverse thermotolerance in grapevines: Vitis vinifera cvs. tends to have SNP-C (-417), whereas wild vines likely have TTC4T(-417) genotypes, thus being more heat-tolerant.

These findings are every important considering that this particular SNP could serve as a marker for rapid screening thermotolerant vine germplasm or as a QTL for breeding. However, there are some points that need the attention of the authors:
1. The literature and databases present VIT_204s0023g00470 (TTC4) as the gene LOC100253049 (protein XP_059592356). It was previously described by Wang et al. (2014, PMID: 26504535) as a member of Group I of the WRKY gene family, as shown here. The authors gave it the name VvWEKY13 and it appears with this name thereafter (e.g., https://www.sciencedirect.com/science/article/pii/S0925521418308_196). They also present data showing that the gene is highly expressed in stamen and pollen tissues of grape, being closely related to Arabidopsis AtWRKY34, which has a role in coordinating gene expression during the formation of the tapetum and microspores. The present article does not demonstrate any of the aforementioned aspects. May this gene have functions other than the thermotolerance presented here? In addition, is it proper to change the name of this gene?

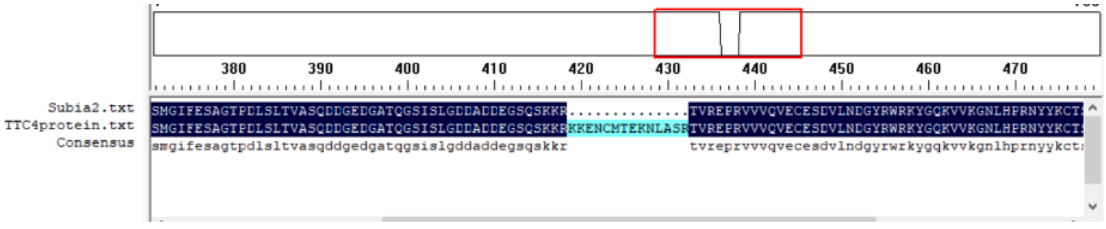
Response: We appreciate your comment. In the revised manuscript, we have cited Wang et al. (2014), who reported that LOC100253049 (protein XP_059592356) encodes a 686-amino-acid protein identified as VvWRKY13. However, VIT_204s0023g00470 encodes a 700-amino-acid protein, indicating that these may represent distinct gene models. In our study, we focused on the thermotolerance function of VIT_204s0023g00470 and named it *TTC4* (Thermotolerance on Chromosome 4). We drew inspiration from previous work on thermotolerance-related genes, such as *TT1* (Thermo-tolerance 1) in African rice (*Oryza glaberrima*), where genetic variations in both the promoter and coding regions contribute to enhanced heat tolerance (Li et al., 2015). While Wang et al. (2014) highlighted the expression of *VvWRKY13* in stamen and pollen tissues, potentially implying additional roles, our research specifically examined the thermotolerance function of the 700-amino-acid protein encoded by VIT_204s0023g00470. Thus, we do not exclude the possibility that this gene may also be involved in other biological processes. We have now included this information and relevant references in the revised manuscript to clarify the nomenclature and functional focus.

Li XM et al. Natural alleles of a proteasome α2 subunit gene contribute to thermotolerance and adaptation of African rice. Nature Genetics. 2015, 47: 827-833.

Wang M et al. Genome and transcriptome analysis of the grapevine (*Vitis vinifera* L.) WRKY gene family. Horticulture Research. 2014, 1: 14016.

2. The definition of VvWKY13 protein in NCBI (NCBI Reference Sequence: XP_059592356.1) is "WRKY transcription factor SUSIBA2 isoform X3 [Vitis vinifera]". Blastp searches reveal high homologies of VvWKY13 with WRKY transcription factor SUSIBA2 from various plant species. How does this correlate with the present results?

Response: We appreciate your comment. According to the NCBI database (LOCUS XP_059592356), the *SUSIBA2* gene was named *VvWKY13* by Wang et al. (2014). However, Sun et al. (2003) reported that WRKY transcription factor SUSIBA2 is specifically expressed in endosperm, and is induced by sugar, playing a key role in starch synthesis. By contrast, our sequence alignment indicates that the *TTC4* studied here is distinct from both *VvWKY13* (*SUSIBA2*) and their reported functional characteristics. Although these genes may share two WRKY domains, our data show that *TTC4* responds to heat stress in grape leaves—an expression pattern and function unlike *SUSIBA2*—thus aligning with the thermotolerance phenotype described in this study.



Sun CX et al. A novel WRKY transcription factor, SUSIBA2, participates in sugar signaling in barley by binding to the sugar-responsive elements of the iso1 promoter. The Plant Cell. 2003, 15: 2076-2092.

Wang M et al. Genome and transcriptome analysis of the grapevine (*Vitis vinifera* L.) WRKY gene family. Horticulture Research. 2014, 1: 14016.

3. Monitoring changes in Fv/Fm can provide insights into the impact of heat stress on plant health and thermotolerance. However, Fv/Fm values may not be the sole indicator

of thermotolerance. Electrolyte leakage was also applied in some cases to verify thermotolerance, but not in all cases/experiments. Is there any explanation?

Response: We appreciate your comment. Previous studies have shown that the OJIP test, which uses F_v/F_m as a key parameter, is faster, more sensitive, and more convenient for assessing plant thermotolerance (including in grape leaves) than measuring electrolyte leakage (Xu et al., 2014; Kalaji et al., 2016). It is widely recognized as a reliable indicator of thermotolerance. In this study, we evaluated the thermotolerance of two natural populations and two hybrid progenies by measuring F_v/F_m values (Fig. 1a, Fig. 7f – 7i). Nevertheless, to address the concern and verify the thermotolerance functions of *TTC4*^{TW}, *TTC4*^{JX}, *HSP18.1*, *APX3*, and *SPL13*, we additionally examined leaf performance and electrolyte leakage under high-temperature conditions. This more comprehensive approach provides stronger evidence supporting these genes' roles in thermotolerance. We have included these methodological details in the revised manuscript.

Xu HG et al. Comparison of investigation methods of heat injury in grapevine (*Vitis*) and assessment to heat tolerance in different cultivars and species. BMC Plant Biology. 2014, 14: 156.

Kalaji HM et al. Chlorophyll a fluorescence as a tool to monitor physiological status of plants under abiotic stress conditions. Acta Physiologiae Plantarum. 2016, 38: 102.

4. Ln. 120-121. In Suppl. Fig. 1b, it appears that there is a notable signal on Chr4, whereas the presence of a significant signal on Chr7 is not obvious.

Response: We appreciate your comment. Using a Mixed Linear Model (MLM) for our GWAS, we identified 15 loci on chromosome 4 and one locus on chromosome 7 that were significantly associated with F_v/F_m values (Fig. 1b-1c). However, the significant signal on chromosome 7 did not have any annotated genes within a 2 Mb region upstream or downstream. Consequently, we focused our analysis on the loci identified on chromosome 4.

5. Lns. 305-328. An in-depth critical comparison with the present results would be more appropriate. The same for Lns. 330-356.

Response: We appreciate your comment. In Lines 305-328 (revised to 331-353), we emphasize the critical role of WRKY transcription factors in plant thermotolerance.

However, many previous studies lacked forward-genetic evidence or required further validation, making it difficult to determine which specific *WRKY* gene is essential for thermotolerance. In this work, we identified *TTC4* using GWAS and confirmed its vital function through overexpression and RNAi experiments. In Lines 330-356 (now 355-381), we cited studies indicating that WRKY factors bind W-box elements to regulate target genes. Yet whether WRKYs directly regulate *HSPs* and *APXs* remained unclear. Our experiments demonstrate that TTC4 modulates grape thermotolerance by directly regulating *HSP18.1* and *APX3* (Fig. 3-4, Extended Data Fig. 5-9).

6. Lns. 486-487. I think the term "haplotype" may not correct here, since there is only one locus (SNP) under consideration. Consider "genotype" instead.

Response: Thank you for the suggestion. We have replaced "haplotype" with "genotype" in the revised manuscript.

7. I also noticed few minor English errors or typos.

Response: Thank you for your helpful note. We have thoroughly reviewed and corrected minor English errors and typos in the revised manuscript.

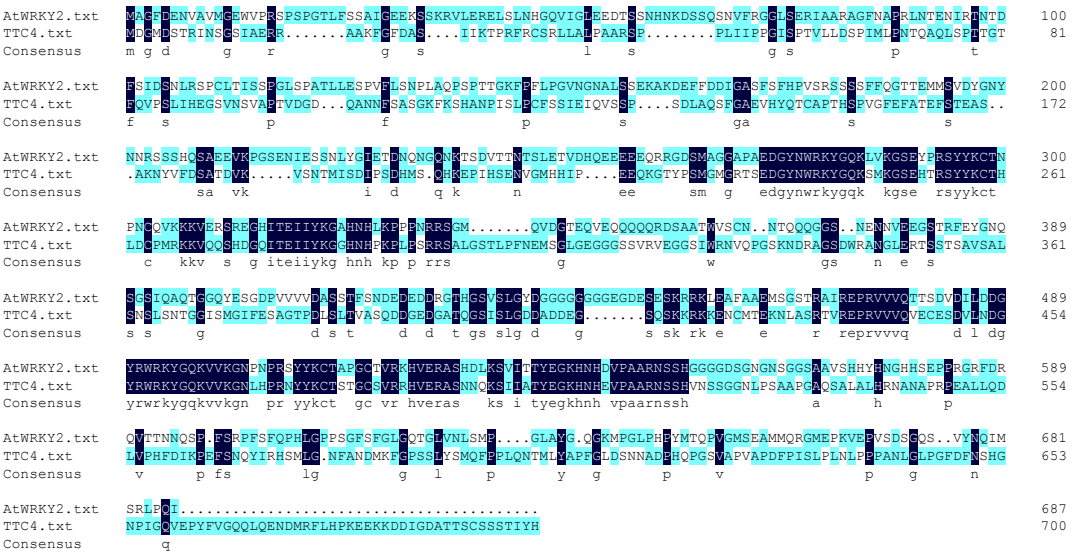
Fig.1g. The phylogenetic tree needs interpretation. How the "similar WEKY proteins" were selected. Any conclusion? Also, the names of the proteins are not informative. Maybe NCBI accession numbers would help.

Response: Thank you very much for your feedback. Following your suggestion, we used OsWRKY2 as an outgroup and constructed a phylogenetic tree for $TTC4^{TW}$ and $TTC4^{JX}$ with proteins showing high sequence similarity. The analysis revealed that both $TTC4^{TW}$ and $TTC4^{JX}$ share the highest homology with WRKY2 from *Gossypium hirsutum*. In addition, we have included the corresponding NCBI accession numbers in the revised manuscript for reference.

Fig. 2h-i. Data for TTC4 conferring thermotolerance to Arabidopsis are not convincing. In Arabidopsis, only phenotypic data and the survival rates are presented. Data from the relative electrolyte leakage, Fv/Fm values, and control (empty vector) are missing. I wonder if Hsp18.1 and APX3 genes were also upregulated? Finally, how likely it is for TTC4 to share the same function in Arabidopsis?

Response: We appreciate your comment. In the original PN40024v1.0 annotation,

TTC4 was predicted to encode a 502-amino-acid protein. However, by re-cloning the *TTC4* gene (*TTC4*^{TW} and *TTC4*^{JX}) using PN40024v4.0 (Velt et al., 2023) as the reference and confirming via RACE, we identified a full-length 700-amino-acid protein (Extended Data Fig. 1a, 3). Transient and stable overexpression assays demonstrated that both updated *TTC4*^{TW} and *TTC4*^{JX} retain thermotolerance functions (Extended Data Fig. 4a, Fig. 2a-2c, Extended Data Fig. 5a, Fig. 2g-2h). Due to time constraints, we have not yet generated *Arabidopsis* lines overexpressing these full-length *TTC4* variants. Additionally, current literature often places emphasis on homologous rather than heterologous transformations. AtWRKY2 and TTC4 share only 26.37% identity at the protein level, and *AtWRKY2* itself is known to function in pollen development and photomorphogenesis (Lei et al. 2017; Wang et al., 2021).



Lei RH et al. Arabidopsis WRKY2 and WRKY34 transcription factors interact with VQ20 protein to modulate pollen development and function. The Plant Journal. 2017, 91: 962-976.

Velt A et al. An improved reference of the grapevine genome reasserts the origin of the PN40024 highly homozygous genotype. G3-Genes Genomes Genetics. 2023, 13: jkad067.

Wang SL et al. WRKY2 and WRKY10 regulate the circadian expression of *PIF4* during the day through interactions with CCA1/LHY and phyB. Plant Communications. 2021, 3: 100265.

Reviewer #3 (Remarks to the Author):

The paper describes the identification of a gene involved in the control of

thermotolerance in grapevines. The authors also identify a putative single nucleotide, fixed for different alleles in Vitis vinifera and in all other wild species tested, that would be responsible for differences in expression that result in differences in thermotolerance. While I appreciated a lot all the functional validation experiments that the authors carried out, I have some serious reservations on their premises, i.e. the genetic analyses and the identification of the putative candidate gene.

The main problem lies in the structure of the identified TTC4 gene. There is no indication in the paper either in the results or in the methods section or in the reporting summary on which reference sequence was used or on which gene prediction and annotation version was used. The gene identifier listed in the paper (VIT_204s0023g00470) would seem to indicate that they used the Vitis vinifera 12Xv0 genome assembly of the strain PN40024 (GCA_000003745.2) and that the gene models refer to the V2.1 gene annotation. The structure of the VIT_204s0023g00470 gene (for which I report hereunder both the predicted protein and the nucleotide sequence of the predicted mRNA) is however substantially different from the one reported here that instead seems to correspond to that of the previous version of the gene prediction and annotation V1.0 where the gene was labelled as VIT_04s0023g00470.

Response: Thank you very much for your critical evaluation. As you noted, we originally identified the *TTC4* gene based on PN40024v1.0 and performed RNA-seq annotation using PN40024v2.0, which led us to characterize a 502-amino-acid form of *TTC4*. However, this version represents a truncated protein. Following your suggestion, we have now cloned the full-length *TTC4* (700 amino acids) using PN40024v4.0 in the revised manuscript.

In the VIT_04s0023g00470 the predicted protein encoded by the gene is 502 aa long as reported in the paper and the ATG starting codon is located on chr4 at base 16666517 on the – strand. 417 bp upstream of it, at base 16666934, the reference sequence of PN40024 contains a C on the – strand that is the last base of a GTAC motif starting at base 16666937. The base at 16666934 is variable among Vitis species as a consequence of a C-T transition. All this matches what is reported in the paper even though there is no reference to any coordinate system. Based on this information the C-T

polymorphism would fall in the putative promoter region of the gene as reported in the paper.

The VIT_204s0023g00470 gene prediction is however different, because it starts with the ATG starting codon much further upstream (on the – strand) at base 16674564, if we look 417 bp upstream we do not find either the expected GTAC motif or the reported G base , so all this makes us conclude that the whole paper was based on the previous gene prediction and annotation version. The VIT_204s0023g00470 prediction produces a predicted protein that is substantially longer that 512 aa with an additional 198 aa at the N-terminus of the protein and most importantly with 2 additional exons and a transcription starting site that is around base 16674777. This is fully supported by the following evidences:

1) the alignment of RNAseq reads that show that the correct gene prediction is not VIT_04s0023g00470 but rather VIT_204s0023g0047;

2) the new prediction performed on the new T2T assembly of PN40024 visible at NCBI (https://www.ncbi.nlm.nih.gov/nuccore/NC_081808?report=graph&v=18127871:18145836;

https://www.ncbi.nlm.nih.gov/protein/XP_059592355.1?report=genbank&log$=protalign&blast_rank=2&RID=YGYAMSM1013) that match VIT_204s0023g0047 and not VIT_04s0023g0047

3) the location of the peaks for the histone modifications that are typical of TSS (H3K4me3 and H3K27ac) that are located in a region between 16674000 and 16675000 bp on chr 4 (Schwope et al. Plant Journal 2021).?

4) Finally and perhaps most importantly the structure of WRKY genes in other species that corresponds to that of the VIT_204s0023g00470 prediction and not of the VIT_04s0023g00470 used here.

Response: Thank you very much for your critical evaluation. Another reviewer also pointed out the same unclarity. In the original manuscript, we cloned TTC4 gene encoding a 502-amino-acid protein based on grape PN40024v1.0. However, as you said, this TTC4 protein only represents a truncated protein. Following your suggestion, we cloned *TTC4* gene encoding a full-length protein consisting of 700 amino acids based

on the updated PN40024v4.0. In doing so, we discovered that our previously cloned 2.3 kb "promoter" region (based on PN40024v1.0) in fact encompassed a large portion of intron 2 and a small section of exon 3 according to PN40024v4.0 (Fig. 5 in the revision).

The implications of these differences in gene structure are of paramount importance for the paper under review:

1) the functional experiments involving the TTC4 gene used a truncated version of the protein, missing 198 aa at the N terminus

2) the identified SNP does not lie in the promoter but rather in the second intron of the predicted gene

3) the promoter that was cloned and used in the paper for functional experiments (that based on the primers provided in Supplementary Table 7 spans a region between bp 1666518 and 16668829) does not correspond to the promoter of the gene but rather to a small part of coding sequence and a large part of the second intron that includes approximately 1.3 kb annotated as a LINE transposable element (that in the grapevine genome are almost exclusively found within introns).

Response: Using PN40024v4.0, we re-analyzed the *TTC4* coding sequence, promoter region, protein function, and SNP variation across 121 grapevine accessions. Furthermore, the identified critical SNP (7631) is located in the intron 2, not in the promoter (Fig. 5). We also separately cloned a 1,479 bp true promoter fragment based on the updated reference. We now present data using the full-length TTC4 protein (700 aa) and distinguish between the actual promoter region and the intronic SNP, aligning our analysis with the updated genome annotation. The revised manuscript incorporates these new findings and highlights how the reviewers' comments substantially improved the accuracy and quality of our study.

So I think it would be extremely important that the authors justify their findings and observation in light of these differences between their evidences and the evidences provided by others on the gene structure and possibly repeat their experiments using the correct protein sequence and promoter sequence. It is not unheard of that regulatory elements of different types (silencers, enhancers, insulators) are found in the introns but this would still imply that the functional experiments performed should be redone

entirely in my mind.

Response: Thank you very much for your critical evaluation, constructive criticism, and thoughtful suggestions. We re-cloned the *TTC4* coding sequences from *Vitis davidii* cv. Tangwei (TW) and *Vitis vinifera* cv. Jingxiu (JX) using 5' RACE based on the updated grape PN40024v4.0 (Extended Data Fig. 3). We found no alternative splicing of *TTC4* in either TW or JX under normal or high-temperature conditions (Extended Data Fig. 3).

Additionally, we assessed the transactivation activities of $TTC4^{TW}$ and $TTC4^{JX}$ in maize protoplasts, and observed similar activities between the two variants (Fig. 1g). We re-examined the subcellular localization of $TTC4^{TW}$ and $TTC4^{JX}$ in tobacco leaves using a transient assay. Our results showed that both $TTC4^{TW}$ and $TTC4^{JX}$ localize to the nucleus under normal and high-temperature conditions (Extended Data Fig. 2).

Furthermore, we re-assessed the thermotolerance function of both $TTC4^{TW}$ and $TTC4^{JX}$ using transient and stable overexpression in grape. Our findings demonstrated that overexpressing either $TTC4^{TW}$ and $TTC4^{JX}$ significantly enhanced grape thermotolerance (Fig. 2a-2c, Fig. 2g-2h). Conversely, RNAi-mediated silencing of TTC4 reduced thermotolerance (Fig. 2d-2f).

We verified that one of the mechanisms underlying thermotolerance divergence among grapevines is determined by a SNP in the *TTC4* intron 2-2, rather than in the promoter or exons. This natural variation—from T(7631) to C(7631)—affects SPL13 binding, resulting in repression of *TTC4* expression. Moreover, the regulatory activity of $TTC4^{T(7631)}$ in intron 2-2 is significantly higher than that of $TTC4^{C(7631)}$ in enhancing gene expression (Fig. 5). Our revised manuscript highlights these new findings, and we believe the functional characterization of *TTC4* and the SNP-T(7631) haplotype offers significant potential for future breeding of heat-tolerant grapevine cultivars.

I have some additional comments on the other genetic analyses:

1. The structure of the population used for GWAS is extremely unusual, being made of different species and interspecific hybrids as well as intraspecific genotypes. The reasons for choosing such a population should be explained.

Response: Thank you for your question. Previous research has shown that *Vitis vinifera*

generally has lower thermotolerance compared to wild species or interspecific hybrids (Xu et al., 2014). Therefore, our diverse grape population, encompassing wild species, interspecific hybrids, and intraspecific genotypes, provides a broad range of phenotypic variation, making it particularly suitable for identifying key thermotolerance genes.

Xu HG et al. Comparison of investigation methods of heat injury in grapevine (*Vitis*) and assessment to heat tolerance in different cultivars and species. BMC Plant Biology. 2014, 14: 156.

2. There is no reference to the genome assembly or gene prediction versions used.

Response: We cloned the *TTC4* genes using PN40024v4.0 as the reference and analyzed the RNA-seq data based on PN40024v4.0. We have clarified this information in the revised manuscript.

3) There is no detail on how the filtering of the SNPs used for the GWAS analysis was carried out. It is surprising that after whole genome resequencing of 121 genotypes belonging to 8 different species, a set of only 2M SNPs to be used for GWAS is identified.

Response: We resequenced the 121 accessions and identified a total of 2,202,663 single nucleotide polymorphisms (SNPs) using GATK software based on the PN40024v4.0 grape genome (Velt et al., 2023). Applying a minor allele frequency (MAF) threshold of 0.05, a missing data threshold of 0.02, and linkage disequilibrium (LD) of 0.2, we filtered the dataset and retained 328,870 SNPs. Subsequently, we performed GWAS using the Mixed Linear Model (MLM) approach in TASSEL (Bradbury et al., 2007).

Bradbury PJ et al. TASSEL: software for association mapping of complex traits in diverse samples. Bioinformatics. 2007, 23: 2633-2635.

Velt A et al. An improved reference of the grapevine genome reasserts the origin of the PN40024 highly homozygous genotype. G3-Genes Genomes Genetics. 2023, 13: jkad067.

4. There is no estimate provided on what fraction of the phenotypic variance for the Fv/Fm is explained by the identified locus on chr 4. This is important to understand if the locus should be considered a QTL or rather a mendelian locus.

Response: Thank you. We have provided a supplemental file (Supplementary Data 1) detailing the fraction of phenotypic variance associated with F_v/F_m. The two SNP markers for *TTC4* are 4_16910407 and 4_16918702, with PVE (phenotypic variation

explained) values of 0.064 and 0.021, respectively—indicating 6.4% and 2.1% of the F_v/F_m variance. Therefore, these markers should be considered QTLs rather than Mendelian loci.

5. The interval identified by GWAS comprises 200 predicted genes. There is no explanation as to how they went from these 200 genes to the 84 considered for expression differences, nor how they went from these 84 to the 6 reported in Fig. 1d. There are other genes in the set of 84 reported that seem to be more upregulated under high temperatures that the 6 identified and VIT_204s0023g00470 does not seem to be the most induced in JX.

Response: Thank you. Using GWAS, we identified 15 loci on chromosome 4 and one locus on chromosome 7, all significantly associated with F_v/F_m—a key indicator of photosynthetic efficiency under heat stress (Fig. 1b-1c). However, the Chr7 signal lacked annotated genes within a 2 Mb region upstream or downstream, prompting us to focus on Chr4, where there are 121 predicted genes, not 200 as initially anticipated. Of these, 97 genes were detected in RNA-sequencing data from the heat-tolerant *V. davidii* 'Tangwei' (TW) and the heat-sensitive *V. vinifera* 'Jingxiu' (JX) under various high-temperature conditions. Our selection criteria prioritized genes upregulated (fold change $\geqslant$1.5) under heat, yielding 16 candidates for further analysis (Supplementary Table 3, Fig. 1d).

In addition, we conducted qRT-PCR on these genes in TW and JX plants exposed to 42°C for different durations (Fig. 1e). Among the resulting data, VIT_204s0043g00820 and VIT_204s0023g00470 were strongly induced in both TW and JX under high temperatures (Fig. 1e). VIT_204s0023g00470 encodes a WRKY transcription factor—a family well-documented in heat stress responses—whereas VIT_204s0043g00820 encodes a putative pantetheine phosphate adenylyltransferase (PPAT) involved in Coenzyme A biosynthesis (Gupta et al., 2021). Hence, we focused on VIT_204s0023g00470 as the more promising candidate for thermotolerance research.

Gupta A et al. Phosphopantetheine Adenylyltransferase: A promising drug target to combat antibiotic resistance. Biochimica et biophysica acta. Proteins and proteomics. 2021, 1869: 140566.

6. It is not clear how the GWAS on the presumed promoter of TTC4 reported at lines 226-229 differs from the initial one.

Response: Thank you. To identify key variation sites within the *TTC4* sequence, we performed an association analysis between genetic variations in *TTC4* and F_v/F_m values using 121 grapevine accessions (Fig. 5a, Supplementary Table 1). This analysis differs from the initial GWAS shown in Fig. 1b and 1c, which was genome-wide. Our targeted analysis revealed two SNPs—SNP (-617) in the promoter and SNP (7631) in intron 2—that are significantly associated with F_v/F_m (Fig. 5a). We found that pro^{TW} and pro^{JX} exhibit similar activities, indicating SNP (-617) does not affect promoter function (Fig. 5e). In contrast, the regulatory ability of $int2\text{-}2^{TW}$ and $pro^{TW}int2\text{-}2^{TW}$ was notably higher than that of $int2\text{-}2^{JX}$ and $pro^{JX}int2\text{-}2^{JX}$, respectively (Fig. 5e). These findings suggest that differences in the *cis*-elements of int2-2 between $TTC4^{TW}$ and $TTC4^{JX}$ are likely responsible for the divergence in *TTC4* regulation. We have emphasized these new results in the revised manuscript.

7. At lines 279-282, the diversity within species (*Vitis vinifera*) cannot be compared with divergence between species (diversity in wild species) so that this difference in diversity cannot be used as evidence of selection acting in vinifera.

Response: Thank you for your comment. We agree that the original discussion (Lines 279-282) was inappropriate. Therefore, we removed this section and omitted any further discussion of "select" in the revised manuscript.