Genetic Interactions and Molecular Evolution of the Duplicated Genes *ICARUS2* and *ICARUS1* Help Arabidopsis Plants Adapt to Different Ambient Temperatures

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REPORT: (The report shows the major requests for revision and author responses. Minor comments for revision and miscellaneous correspondence are not included. The original format may not be reflected in this compilation, but the reviewer comments and author responses are not edited, except to correct minor typographical or spelling errors that could be a source of ambiguity.)

TPC2018-00841-RA 1st Editorial decision – *declined* Dec. 9, 2019

Overall, the editors and reviewers judged that the topic and approach were interesting and relevant to the readership of The Plant Cell. A better understanding of the evolution of natural epistatic variation would be an important contribution to the field, especially if supported by functional studies of the interacting genes. However, the reviewers identified a number of shortcomings. There was a consensus that the presentation was not clear or focused. The authors pursued several interesting but unconnected components of research related to ICA1 and ICA2. As a result, the overall message and final interpretation were not strongly presented. Reviewers raised a number of concerns with the evolutionary analyses of gene duplication. Reviewer 1 posed several relevant questions about the function of alleles and their frequency, with implications for understanding temperature sensitivity and climate associations. Reviewer 3 noted limitations to the phylogenetic analysis along with suggestions for improving the evolutionary inferences of gene duplications, including the use of Bayesian or ML phylogenetic analyses. Finally, all reviewers identified problems with the functional studies and their interpretation. In particular, details of expression and transgenic studies were lacking, temperature sensitivity experiments were not balanced, and the analyses could be improved using mixed models. In the aggregate, these concerns preclude the acceptance of the manuscript.

RESPONSE: We are aware that the manuscript includes two different lines of research corresponding to the functional and evolutionary/adaptive aspects of the *ICA2/ICA1* interaction, and we agree that the parallel description of both components used in the previous version was not fully clear and, sometimes, even confusing. In the new version we have rearranged the various questions addressed in the study, describing first the functional aspects and thereafter the evolutionary research. In addition, we have extended the functional results by including two new experiments (cytometry measurements of cell DNA content, and DNA damage sensitivity) and by describing in more detail the fine mapping and the interaction between *ICA2* and *ICA1* natural alleles (which also required some additional plant measurements to obtain the graph with the L*er*/Don interaction that has now been added). Furthermore, following the suggestions of several Reviewers (see below), we have reduced the speculative paragraphs on adaptation and carefully remade and revised the evolutionary analyses of gene duplications. We believe that these major changes focused our study and made clearer the messages.

---------- Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2018-00938-RA Submission received Feb. 26, 2019

Reviewer comments on previously declined manuscript and **author responses:**

Reviewer #1:

In this study, Mendez-Vigo et al. has carefully dissected a QTL and showed that there is within-QTL epistasis between two paralogous genes, *ICA1* and *ICA2*. In 2015, the authors (Zhu et al. 2015) identified a loss-of-function *ICA1* allele in accession Don-0, which confers abnormal phenotypes under heat stress. Here the authors found more complex epistasis within the same QTL: Under functional *ICA1*, allelic variants of *ICA2* do not affect phenotype. With *ICA1* loss of function, ICA2-Ler remains normal and ICA2-Don-0 has abnormal growth under heat stress. This is a two-loci epistasis, where an abnormal phenotype happens only when an accession has homozygous loss-of-function ICA1 and ICA2-Don-0 (presumable with weaker protein function than ICA2-Ler). The authors then investigated the copy numbers and genomic locations of ICA genes across plant phylogeny as well the *ICA2* global allelic distribution in *Arabidopsis thaliana*.

The amount and quality of work presented by the authors is great, but the main message of this manuscript is not very clear. Here we have a system where two paralogs have redundant functions, and abnormal phenotypes happen when one (ICA1) has lost function and the other (ICA2) has weak function: the Don-0 accession is unfortunate to be homozygous for both.

Point 1. After identifying the two genes, there are two possible directions: (1) Investigate the molecular function of both genes, especially the protein differences between ICA2-Ler and ICA2-Don-0; (2) Investigate the evolutionary fate of ICA. While the authors performed the latter, the analyses do not catch the main point. For example, the whole section about the broad phylogenetic relationship of ICA does not relate much to the main theme of ICA1-ICA2 epistasis or functional redundancy.

> **RESPONSE: This raises the same point described by the Editors and explained above. We believe that combining both functional and evolutionary results allows an integrated and better understanding of natural variation, which is uncommon in scientific literature. As described above, in the new version, we have separated both research lines by moving the whole section of intra and interspecific (phylogenetic) analyses to the end of Results. In addition, we balanced both components by reinforcing the functional aspects.**

Point 2. In the INTRODUCTION, the paragraph from line 122-145 also seems independent from the other paragraphs. The authors especially distinguish recent vs. young duplication events, but such an issue does not seem to be the main topic of this study.

> **RESPONSE: We agree that the distinction between recent *vs.* young duplications is not a main topic of our research. To avoid this emphasis, we have now reduced this paragraph by removing such distinction.**

Point 3. For evolutionary analyses, more important questions should be answered. For example, the weak ICA2-Don-0 allele is likely deleterious if ICA1 has lost function, but why is this allele the majority and the ancestral form (Figure 5 and Line 314)? Maybe in most cases ICA1 retained the ancestral function, and the weakening-of-function of ICA2-Don-0 evolved neutrally? In that case, why does the derived Don-Ler allele restore the ancestral protein function?

> **RESPONSE: We agree that understanding the fitness effects that maintain *ICA2* natural variation is an open and interesting question. However, given the diverse pleotropic effects of *ICA2/ICA1* variation at the cellular and organism levels, as well as the lack of knowledge on the precise molecular/regulatory functions of *ICA* genes, it is hard to speculate on it. In addition, given the strong genotype by environment interaction displayed by *ICA1/ICA2* genetic interaction, answering these questions will require true estimates of fitness effects under field or natural conditions. For these reasons, and following the suggestion from Reviewer 3 on this point, we have rewritten and shortened the Discussion section dealing with this question to avoid speculation (lines 471-485 of former version).**

Point 4. One of the major results is the dissection of a ~400 kb QTL, which is truly hard work using a large sample size. However, all the results are squeezed into the first paragraph of the RESULTS and Figure 1B. The authors merely show the conclusion without detailed results of how they reach such a conclusion. Detailed line-by-line comments are in the following section. I also expect to see a reaction norm plot to show epistasis between the two loci.

RESPONSE: We are very glad that Reviewer 1 appreciates the effort and relevance of this result. Following his/her suggestions we have now added a reaction norm showing the strong epistasis between L*er* and Don-0 alleles at both loci. In addition, we have extended the description of results in this section following the line-by-line suggestions described below.

Point 5. For the last section where the authors examined phenotypic effects of transgenic lines, properly designed mixed-model ANOVA tests are required.

RESPONSE: We agree that this statistical test was not appropriate, and thanks again to Reviewer 1 for pointing it out. We have now tested the phenotypic differences using mixed-model ANOVAs, which has been included in the M&M. New statistical analyses show that phenotypic effects of transgenic lines are similar to those reported in the previous version, except for a small difference in flowering time, which is not significant now when testing properly. This has been corrected in the text, and did not affect any of our main conclusions.

Point 6. Line 231-233: At2g32320 from Ler or Don-0 are transformed into Col *ica1-2*. The Col *ica1-2* genomic background still has a functional form of At2g32320 (similar allele to Don-0 At2g32320). So it's natural that transforming Don-0 At2g32320 into Col *ica1-2* won't show a different phenotype. So the logic in line 231-233 seems a bit strange.

RESPONSE: The reason for the conclusion drawn in lines 231-233 comes from the differential behavior (phenotypic complementation or not) between the two transgenes of the same gene (*At2g32320*) from L*er* and Don-0, which we could expect to behave similarly if they were not *ICA2* (the Col *ica1-2* genetic background carries a functional form of *At2g32320* in both types of transgenic lines). However, both *ICA2* transgenes behave differently and their phenotypes are similar to those observed for *ICA1/ICA2* genetic interaction in the mapping population (a single copy of *ICA2*-L*er* is able to restore normal growth at high temperature). This probably was unclear again due to the concise description of the mapping and the interaction between L*er* and Don-0 alleles in *ICA1* and *ICA2*. As indicated above, we have now added more detailed information, including a graphical representation of the genetic interaction, as suggested by the Reviewer. In addition, this sentence in lines 231-233 has been rephrased and explained to avoid confusion.

Point 7. Line 288-289: The lack of expression difference between natural accessions may be caused by different cis-regulatory compensated by different trans-regulatory variation. Measuring allele-specific expression in an F1 may be a good way if the authors want to identify cis-regulatory changes. I do not think comparing expression levels of lines transformed with ICA2-Ler vs. ICA2-Don-0 is useful here. Here the authors want to solve "whether natural alleles of ICA2-Ler and ICA2-Don-0 differ in expression". Since both positional effect and the promoter used affect expression, "whether the two set of lines differ in their expression or not" does not answer the question. The authors should also remind us what promoters were used. Are they native promoters or 35S? The expression was measured in which stage and which tissue?

RESPONSE: We fully agree with the Reviewer that both positional effects and the promoter used affect the expression of transgenes. However, the genomic constructs of *ICA1*-L*er* and *ICA2*-Don-0 alleles were developed to include the native promoters from L*er* and Don-0 and therefore, the promoter effects are those produced by cis-regulatory polymorphisms. Positional effects will also contribute, but to reduce these effects we used a relatively high number of independent homozygous lines (8 per construct).To avoid misunderstanding, we have now indicated the promoter of the constructs in the Results section, since in the previous version of the manuscript this information was included only in the M&M. In addition, we have rewritten several sentences of this section to make it clearer.

Point 8. Line 323-333: The connection with climatic variables is weak. The direction of environmental association is also opposite to expectation - while ICA2-Don-0 is the allele conferring developmental abnormality under high

temperature, it is instead found in regions with higher temperature fluctuation. The allele distribution has no association with mean annual temperature or the temperature when the seed geminated either.

RESPONSE: We agree that the association with climatic variables is not a demonstration of a role in climatic adaptation, but the association with specific climatic variables is significant and supports a relevant hypothesis. We do not find that the association of *ICA2*-Don-0 alleles is opposite to expectation because the effect of *ICA1/ICA2* genes is variable upon temperature (genotype by environment interaction). In the previous study (Zhu et al., 2015) we showed that the strong effect caused by *ICA1*-Don loss-of-function allele at high temperature (which we now know is due to the interaction of both *ICA1* and *ICA2* genes) is reversible and quantitative, depending on temperature. Therefore, under natural conditions, *ICA2* effects likely depend on daily temperature fluctuations more than a fixed temperature. We have now explained this better in the corresponding section of the Discussion, but following the suggestion of Reviewer 3, we have also reduced the Discussion section on climatic adaptation to avoid excessive speculation.

Reviewer #2:

This is an interesting study, following up on previous work on this genomic location and the *ICARUS1* gene presented in Zhu et al. 2015 and Mendez-Vigo et al. 2016. Briefly, by fine-mapping a naturally occurring phenomenon of reduced growth at high temperatures in a cross of two accessions, the authors define that not one, but two interacting homologues of the ICARUS tRNAHIS guanylyltransferases are involved. They characterise the interaction between the two loci (synergistic epistasis) and then different alleles at the newly identified *ICA2* locus. For this they predict substantial protein structural differences but find no clear evidence of expression difference. They follow the evolutionary history of this pair of genes throughout the land plants, observing intra-genic duplication of the Thg1 domain as well as gene duplication specific to the Brassicaceae and Camellinae at a macro-evolutionary scale. Finally, they then analyse the microevolutionary scale, looking at the global distribution of these haplotypes, and find significant correlation to temperature variables within the climate.

Broadly, the genetic interaction analyses seem to me excellently done, and the evolutionary analysis is interesting, and for these the authors' claims are supported. However, the conclusions about the function of ICARUS2 are, I feel, not supported, and form a key part of the Discussion. On the basis of the genetic interaction between *ICARUS2* and *ICARUS2*, their homology and similar plant-scale growth phenotypes, the authors claim that ICARUS2 must be functionally equivalent to ICARUS1. The logic is good but is only a hypothesis - I would recommend major revisions:

Point 1. That at least some of the experimental evidence presented for ICARUS1, involvement in cell-cycle and DNA-damage repair in Zhu et al. 2015 is shown for ICARUS2, such as flow-cytometry measurements and first-leaf assays. These are still not assays of molecular mechanisms, but are at least equivalent to what is known about ICA1. Given the authors have an *ica2* mutant, and transgenic lines complemented with the Don/Col and Ler ICA2 alleles, the particular tests mentioned here should not be too difficult, and would greatly strengthen their case.

RESPONSE: Following the suggestion of Reviewer 2, we have analysed transgenic lines for *ICA2*-L*er* and *ICA2*-Don-0, as well as *ica2-1* mutant and control lines for both assays: measurements of cell DNA content by flow cytometry; and measurements of DNA damage sensitivity by the first leaf assay. The results of these two new sets of data have been added to various sections of the manuscript and, as indicated by the Reviewer, we believe that they have substantially reinforced our functional interpretation of *ICA2*.

Point 2. The figures are generally very well presented, but there is so much data that some of the subpanels are too small (certainly in the PDF I have to review). This is partly a question for the Editor, but I've made several suggestions below to make them simpler and clearer. In particular, I don't think Fig. 4 is as important to the main text of the paper as the other figures, even though it is very attractive. I would suggest moving it to the supplemental data, if it would allow the other figures to be presented at a larger scale.

RESPONSE: We are glad that Reviewer 2 liked the presentation of the figures and we agree that some figure panels were rather small. As described below in the minor points of Reviewer 2, we have now increased the size of several Figures. However, we think that Figure 4A is very important to describe the duplications of *ICA* genes in *Brassicaceae* and therefore should not be moved to the Supplemental section. We agree that Figure 4B could be moved to the supplemental section, but this would ask readers to assume microsynteny in the *ICA1/ICA2* region,

which although expected, needs to be demonstrated. For this reason, we have left Figure 4 (Figure 6 of the new version) in the main text, but we will move any panel to the supplemental section if the Editors and/or Reviewers still consider it convenient.

Point 3. Abstract: "Analyses of transgenic lines in different backgrounds demonstrate multiple ICA2 functional effects, supporting that ICA2 structural polymorphisms underlie this natural variation." Section before comma - multiple phenotypes? Core function uncharacterised. Section after comma is not a logical consequence of this, nor very well supported. Perhaps new sentence "ICA2 structural polymorphisms likely underlie this variation". Or delete entirely.

RESPONSE: We agree that this sentence was confusing, since we did not aim to connect both parts of the sentence as logical cause and consequence, but simply as two results coming out from the detailed analyses of transgenic lines. We have now rephrased this sentence according to suggestions from Reviewers 2 and 3 (see below). In addition, we think that the experiments added to the new version of the manuscript provide the lacking characterization of *ICA2* to support the first part of the sentence.

Reviewer #3:

In their manuscript entitled 'Genetic interaction and molecular evolution of the duplicated genes *ICARUS2* and *ICARUS1* confer growth adaptation to ambient temperatures in Arabidopsis', Mendez-Vigo et al. provide evidence for an epistatic relationship between previously characterized *ICA1* and uncharacterized *ICA2* that show natural allelic variation in *Arabidopsis thaliana*.

Point 1. The data presented make a compelling case that ICA1 and ICA2 interact to regulate aspects of growth and development, some of which were demonstrated to be temperature-dependent. However, since not all experiments were conducted at both 21 and 28 °C (e.g. line 190-235), I would be careful in concluding "the temperature-dependent developmental phenotypes of (the) Don-0 accession are caused by natural alleles in two homologous genes encoding for Thg1 proteins" (line 233-235)."

RESPONSE: We agree with Reviewer 3 that, for most experiments, we presented only results at 28 °C. However, most of them were done at both temperatures but not included in the manuscript for simplification. To support our conclusion, in the new version we have added the description of phenotypes for several of these analyses at both temperatures, 21 and 28 °C, including the growth defects of microRNA lines and transgenic lines, as well as the measurements of DNA content by flow cytometry in transgenic and mutant lines.

Point 2. The weakest part of the paper is the analysis of ICA-like gene duplications across land plants. It is the norm to infer the timing of gene duplication events using a phylogenetic approach, i.e. to align all ICA-like genes from species across the taxonomic group of interest as an input to building a gene tree. The topology of the gene tree then allows the inference of gene duplications based on relationships, but not number of genes per se. Fig. 3 appears to be drawn from the Phytozome representation of the land plant phylogeny, although no citation is given; there are similarly no citations given for age estimates of major diversification events within the text. Fig. 4 then shows a similar phylogeny, but this time apparently reconstructed using ICA-like amino acid (?) sequences. Fig. 4 was confusing in that the number of ICA-like genes per species were apparently given after the species name, but then some of the species were represented multiple times. Why weren't all the ICA-like genes for each species used to construct a gene tree? Also, the Neighbor Joining approach is no longer an acceptable method for constructing phylogeny as it is based on similarity rather than modeling both divergence and convergence. Finally, the description of diversification and duplication events was not accurately written. For example, monocots and eudicots are not considered basal angiosperms, as alluded to on lines 246-247. I would suggest having a single figure with an ICA-like gene tree based on a nucleotide alignment and a Bayesian or Maximum Likelihood analysis that will allow a more accurate timing of both tandem and non-tandem duplication events.

RESPONSE: We agree with the Reviewer that this section was not accurately written, contained several typing errors in Latin names, lack of references, and more importantly, we used an inappropriate method for the construction of the phylogenetic tree. In the new version, we have addressed all these comments following most of Reviewer's suggestions. We have recalculated the ICA tree based on protein sequences using the ML method. Figure 4A has been remade, including not only the new tree, but also giving colors and formats to species names to integrate the results of the ML tree with the results of the structural analysis shown in Figure 3. The terminology of ICA genes has been carefully described in the legend of Figure 4. Phytozome reference and citations for age

estimates have been included. The whole section of the Results has been rewritten to correct errors and inaccuracies, as well as to show the relevance of both analyses used to find and date ICA duplications. We agree with the Reviewer that the common way to infer duplications is to make a phylogeny tree. However, many studies construct such trees using available sequences from gene/genome sequencing projects that are often incomplete. Therefore, often, it is not really known how many copies of the gene contain each species but only how many genes are available. For this reason, we used whole genomes sequences with good quality and well-annotated to determine precisely how many copies of the gene are present in each genome, their genome location and domain structure. As a second step, we have done the ML phylogenetic tree to infer mainly the more recent duplications found in Brassicaceae. The ML tree is in agreement with the analysis of gene copies and protein domain structure, and we think that both analyses complement the addressed questions and reinforce our conclusions. For this reason, we prefer to keep both figures in the manuscript. However, as indicated above in relation to comments from Reviewer 2 on this same Figure 3 (current Figure 5 in the new version), we will move it to supplemental information if the Editors and/or Reviewers still consider it convenient.

Point 3. Line 118: are you implying both tandem and non-tandem duplication events? Perhaps this could be made clearer.

RESPONSE: The precise type of duplications occurred during evolution of *A. thaliana ICA* genes are unknown, but several possibilities could account for it, involving or not tandem duplications. For this reason, we think that in the Introduction it is better to keep the description of genes without speculations on hypotheses. In addition, understanding the evolution of *ICA* genes of Arabidopsis is one of the goals of our study, and these possibilities have been now addressed in the Discussion.

TPC2018-00938-RA 1st Editorial decision – *accept with minor revision* **March 21, 2019**

On the basis of the advice received, the board of reviewing editors would like to accept your manuscript for publication in The Plant Cell. This acceptance is contingent on revision based on the comments of our reviewers. In particular, please clarify the details in Figure 1B and the statistical methods associated with reaction norm plots in Figure 3 (see reviewer 1 comments). In addition, please consider line editing in response to the minor comments from all reviewers.

---------- Reviewer comments:

[Provided below along with author responses]

TPC2018-00938-RAR1 1st Revision received **March 29, 2019**

Reviewer comments on previous submission and **author responses**:

Reviewer #1

The authors have greatly improved this manuscript. Below are some other comments I hope the authors can address:

Point 1. One thing still puzzles me. Figure 1B shows 33 recombinant F2 plants, whose genotypes and F3 progeny phenotypes were analyzed. But I do not see any double homozygotes in repulsion phase (Ler homo in one and Don-0 homo in the other locus) among the 33 F2. How are the data in Figure 1C obtained? Did the authors picked the two F2 family (labeled with * in Figure 1B) and further genotype many F3? It is worth using at least one sentence in the Results to clarify this. Part of it is first mentioned in line 182-185, but maybe clarify it earlier.

RESPONSE: Following the advice of Reviewer 1, we have added a sentence to the Results section (page 7, lines 181-184 of the new version) describing how this analysis of Figure 1C was done. In addition, we have also added a paragraph in the Methods section (page 21, lines 588-597) describing precisely how these plants were obtained and analysed.

Point 2. About the statistical tests for Figure 3: Thank you for performing this test. If I understand correctly, for each trait, this test was done separately for each temperature, and the asterisks indicate the difference between Ler and

Don-0 alleles in each temperature. I would however like to see a test that incorporates temperature into the model: transgene (fixed), line (random, nested within transgene), temperature (fixed), and transgene-by-temperature interaction (fixed). Then I would like to see the differences among the four groups (2 transgenes x 2 environments) within the interaction term. Tests such as Tukey HSD test (implemented in most statistical software I believe) could then be used to quickly look for differences among the four groups.

RESPONSE: Following the Reviewer's suggestion, we have applied these mixed models including the four factors to test simultaneously the effect of transgene, temperature, line, and the interaction between transgene and temperature. These analyses have been now included in the description of the results of Figure 3 (page 12, lines 311 and 317). The description of the statistical tests has also been added to the Methods (page 26, lines 714-719) and to the legend of Figure 3 (page 39, lines 1023-1026 of the file with changes highlighted). We have also applied the Tukey HSD test to contrast the differences between the four groups of lines, and this information has been added to Figure 3 to replace previous statistical tests carried out at different temperatures separately. Overall, these analyses show a similar output as previously presented, and they are in agreement with the general conclusions. Only the expression level of *ICA2* shows now an effect of temperature that was not significant in previous tests. We interpret this temperature effect mainly as consequence of positional effects on *ICA2* transgene insertion. This information has also been added to the new version of the manuscript (page 12, lines 320-330). As pointed out above, these analyses are now described in the Results, Methods and the legend of Figure 3

Point 3. Figure 1B. If I am not mistaken - in the third light blue chromosome cartoon graph (the chromosome with two separate dark purple regions showing ICARUS1 and ICARUS2), on its most left hand side, there is a vertical black line showing a marker (the small black vertical line directly above the "# of rec" text). According to the scale of enlargement, there should not be a marker there, right? Because on the right hand side of the same chromosome there's no marker.

RESPONSE: Reviewer is correct; we have removed this vertical black line in the new Figure 1.

Reviewer #2:

Thanks to the authors for the changes made. The rearrangement of the structure and figures make the manuscript a much clearer read, and the new functional analyses support the story well.

Reviewer #3:

The manuscript is much improved. I only have a few small outstanding comments.

Point 1. Line 467: "Camelineae duplication is older than that of Brassiceae". The ML tree actually suggests that the gene duplication event giving rise to two Camelineae genes occurred at the base of Brassicaceae, and then was lost in the Brassiceae (and other tribes outside Camelineae if we don't infer inadequate sampling). Thus, I wouldn't refer to a "Brassiceae duplication", but rather a "Brassicaceae" duplication."

RESPONSE: We understand the argument of Reviewer. However, taking into account that Brassicaceae is one of the largest and most diverse plant families, including 51 different (and some) controversial tribes (see Huang et al., 2015; Mol Biol. Evol. 33: 394), together with the low number of well annotated genomes available (the 11 Brassicaceae species included in our study), we consider that it is cautious not to speculate further on *ICA* duplications. For this reason, we prefer to refer to "Camelineae duplication", which describes its current restricted presence in this tribe.

TPC2018-00938-RAR1 2nd Editorial decision – *acceptance pending* **April 5, 2019**

We are pleased to inform you that your paper entitled "Genetic interaction and molecular evolution of the duplicated genes *ICARUS2* and *ICARUS1* confer growth adaptation to ambient temperature in Arabidopsis" has been accepted for publication in The Plant Cell, pending a final minor editorial review by journal staff. At this stage, your manuscript will be evaluated by a Science Editor with respect to scientific content presentation, compliance with journal policies, and presentation for a broad readership

Final acceptance from Science Editor **April 12, 2019**