The Peroxidative Cleavage of Kaempferol Contributes to the Biosynthesis of the Benzenoid Moiety of Ubiquinone in Plants

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Review timeline:

TPC2018-00688-BR	Submission received:	Sept 7, 2018
	1st Decision:	Oct. 3, 2018 *accept with minor revision*
TPC2018-00688-BR1	1st Revision received:	Oct. 12, 2018
	2nd Decision:	Oct. 18, 2018 *acceptance pending, sent to science editor*
	Final acceptance:	Nov. 13, 2018
	Advance publication:	Nov. 14, 2018

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.)

TPC2018-00688-BR 1st Editorial decision – *accept with minor revision* Oct. 3, 2018

We have received reviews of your manuscript entitled "The Peroxidative Cleavage of Kaempferol Contributes to the Biosynthesis of the Benzenoid Moiety of Ubiquinone in Plants." 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 consider the following:

Overall the reviewers and myself were quite enthusiastic and complementary on the manuscript and felt it would make an excellent breakthrough report. Some issues were raised that I felt could be dealt with in revision and if fully done, would not require re-review as long as I found them appropriate and complete.

1. All your data is reported for a single mutant allele. *TPC* generally requires more than a single mutant allele to be used for studies or if a single is used that it be complemented to confirm the role of the gene. However, in this case these are well characterized mutants and, perhaps more importantly, the series of mutant loci are internally consistent, *4-3cl*, *chi* and *f3h* impact phylloquinone levels while later mutants do not. However, this is not readily clear from the paper, as noted by Reviewers 1 and 3. I think this can be addressed with a succinct paragraph indicating the well characterized nature of the mutants, that the combined data are internally consistent, mutually supportive etc., that the body of evidence supports the conclusions despite a single allele being used. I presume you can capture these ideas succinctly in an additional paragraph the will help the general readership follow these lines of logic.

2. Reviewer 3 raised a point about the truncated nature of the network shown, with only UQ and flavonoid genes included it may be misleading. The suggestion for using a cutoff for inclusion of other genes (and the one's shown) in the network seems reasonable. It may be that doubling or tripling the number of genes shown in the figure will provide a more representative overview.

3. The third point raised by Reviewer 3 is a good one and addressing it will add further to the indicated section of the discussion and also abstract, to further highlight the truly novel nature of the findings!

The other minor comments by the three reviewers are also potentially useful and should be assessed and considered. When you submit your revised manuscript if you could include one with changes tracked, to make it easier for me to assess the changes made, I would appreciate it. I look forward to receiving it.

See attached file for minor comments on increasing the font sizes systematically in all figures. You should also use freeze panes option on the header of the Excel pages and provide a legend for that table so that the reader understands the headers.

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

[Reviewer comments shown below along with author responses]

TPC2018-00688-BR1 1st Revision received **Oct. 12, 2018**

Reviewer comments and **author responses**:

We sincerely thank the Reviewers for their time and for their constructive comments.

Reviewer #1:

The manuscript by Soubeyrand provides important information concerning an essential plant metabolite that has surprisingly revealed very little attention. The experiments are well planned and executed and the manuscript reads very well. I have one major (and no other) criticism of the manuscript which is that the data are based on single "mutant alleles" I would have liked to have seen a second allele or complementation to support their work. I do see that using mutants of the same pathway is similar supporting evidence. However, while I realise this is probably an editorial decision I would be much happier if further genetic evidence were provided to support their study.

We fully acknowledge that T-DNA, EMS and X-ray generated mutants have the potential to contain secondary mutations, and therefore that it is usual practice to study several mutant alleles of the same gene, in particular in the case of a newly characterized gene. In our study, however, all the selected mutants had been previously characterized and shown to correspond to genuine null alleles; T-DNA lines had been shown to be fully complemented after re-transformation with their cognate wild-type allele, and the EMS/X-ray mutants were backcrossed. More importantly, the combined data obtained with these knockout lines are mutually consistent, that is: all the mutants corresponding to enzymes located upstream of F3H and including the later (*4-cl3*, *chs* and *f3h*) negatively impact ubiquinone level, while those located downstream this enzyme (*f3'h* and *dfr*) do not. It is also noteworthy that the data obtained with the Arabidopsis *f3h* knockout are congruent with those of the tomato *f3h* mutant. We have re-worked the results section to clarify these points.

Reviewer #2:

This is a fascinating study. It shows that the flavonoid biosynthetic pathway in plants serves a novel purpose as a branchpoint to generate 4-hydroxybenzoic acid (4-HB), the ring precursor of coenzyme Q (CoQ or ubiquinone), an essential component of mitochondrial respiration. The authors combine the use of Arabidopsis flavonoid biosynthetic mutants with clever feeding assays, isotopic labeling and chemical rescue experiments to show that kaempferol is converted to 4-HB. Plants engineered to produce high levels of flavonoids, or plant cells treated with kaempferol exhibit increased levels of CoQ. Moreover, the authors show that it is the B-ring of kaempferol that is converted to 4-HB, and this process is dependent on heme peroxidase activity. These findings answer a long-standing puzzle. The peroxisomal pathway that oxidizes p-coumarate to 4HB accounts for roughly half of the CoQ synthesized from p-coumarate. The results show that synthesis of kaempferol from p-coumarate in the cytosol functions as an independent pathway producing 'the other half' of p-coumarate derived 4-HB. Furthermore, this pathway is also likely to operate in mammalian cells. The authors discuss the implications of their study on the recent findings that exogenously supplied kaempferol boosts CoQ levels in cultured mammalian kidney cells. The following minor suggestions are made to help increase the clarity of the presentation:

1. Figure 1A is plotted amount of 13C6-ubiquinone versus time. However, aside from the time zero point, there are just two time points (2 and 3 hours). These two points do not seem to form a slope consistent with the line going through zero time. This is reflected by poor connection of the line from time zero and 3 h with the point at 2 h. It might be more informative to plot all measurements as a scatter plot, and delete the time course line. Also, much of the text in Figure 1B and 1C is so small it is almost illegible. Please enlarge.

As suggested, we have re-drawn Figure 1A as a scatter plot and used colors to help distinguish the data points. Figures 1B and 1C has been entirely re-drawn. Font size has been increased for all figures [we sincerely apologize for the lack of legibility of our initial figures].

2. It is not clear what the purpose is served by the line connecting the points in Figure 2C. In this case it is not a time course, and the points can be shown without the line. Here again, it would be useful to plot the data as a scatter plot instead of dynamite plots.

Actually, *sensu stricto* Figure 2C is a time course experiment: It represents the change in ubiquinone content in tomato fruits as those progress through ripening, from green to red ripe (breaker+ 7). We agree that the lines that extrapolate ubiquinone content between the different stages of ripening in WT and transgenic fruits are not crucial. We have removed them in this new figure version.

3. The authors explain that mutants lacking FLS activity are not available due to the many FLS homologs present in plants. Are inhibitors available that affect FLS activity? This would provide a chemical approach to test that this activity is essential for conversion of dihydrokaempferol to 4-HB and CoQ.

We do not know of any specific inhibitor of FLS. Such a lack of specificity is a major issue, because FLS belongs to the large family of iron-2-oxoglutarate-dependent dioxygenases (66 members in Arabidopsis), the non-specific inhibition of which would have devastating effect on overall cell metabolism.

4. The identification of peroxidase activity as essential for conversion of kaempferol to 4-HB is very interesting. Perhaps the authors could elaborate on possible candidate genes for the peroxidase activity present in plant cells, and touch on the nature of possible candidates in kidney cells too.

We have indeed identified a heme-peroxidase candidate -called SAPX (At4g08390)-in the gene network of COQ1, COQ8 and COQ9 [you can see it in Supplemental dataset 1]. Pilot ubiquinone analysis in a corresponding confirmed knockout line did not reveal any statistically significant differences as compared to wild-type controls. We have included these data as a Supplemental Figure 'for review only' as these are essentially negative results. Given the known substrate promiscuity of plant heme-peroxidases, we think that several of these enzymes most probably contribute to the cleavage of kaempferol. We do not feel sufficiently qualified to comment on possible peroxidase candidates in kidney cells. With that being said, the occurrence of heme-dependent peroxidases acting on a wide-range of substrates is well documented in mammalian cells (e.g. PMID 18331199). The predominant peroxidase in human kidney cells, however, appears to be glutathione peroxidase 1 (GPX1). The enzyme is selenocysteine-dependent, not heme-dependent.

Reviewer #3:

Soubeyrand *et al.* reports a novel pathway of ubiquinone (UQ) biosynthesis through the flavonoid pathway in Arabidopsis. The same group previously demonstrated that Arabidopsis can synthesize UQ from both tyrosine and phenylalanine. The current study conducted genetic analyses of various mutants combined with bioinformatic analyses, ^{13}C-labeled substrate feeding experiments, and enzyme assays and uncovered that the B-ring of kaempferol can be incorporated into 4-hydroxybenzoate and the head group of UQ. It has been well documented that various specialized metabolic pathways evolved downstream of primary metabolism. In contrast, the current study revealed that a specialized metabolic pathway (i.e. flavonoid biosynthesis) can contribute to the production of a primary metabolite (i.e. UQ). Thus, the current finding is intriguing, conceptually novel, and nicely fits to the scope of Breakthrough Reports. The manuscript is overall well written and organized. A logical series of experiments were conducted and presented and their results nicely support the main conclusion of the study. My comments are, I believe, largely editorial but the first three points are critical points that need to be address to fully backup this important finding. Major comments: Line 115, 162 -Are these "knockout alleles" confirmed null mutants? This information is critical for properly interpreting metabolite data. Please clearly describe in the result or method where readers can find the actual data confirming null knockout mutations (or there are some leakiness).

Yes, we confirm that the Arabidopsis mutants used in this study have all been previously shown to correspond to genuine null alleles of their cognate enzymes. The corresponding references have been included in the method section. The tomato *f3h* mutant (alias *are*), on the other hand, is 'leaky', which we have stated in the result section.

Figure 1B is misleading as only flavonoid metabolic genes are shown. Readers cannot evaluate how strongly the UQ and flavonoid biosynthetic genes interact. Although flavonoid and UQ genes may be highlighted, other genes in the network should be also shown (e.g. using a certain cut off). The same critique applies to Figure 1C.

The reviewer is correct: flavonoid biosynthetic genes are not the sole interactors of the query genes. It was definitely not our intention to mislead the reader regarding the general architecture of our gene network reconstruction; we sincerely apologize. We have entirely re-drawn Figure 1B to show all the co-expressors -out of the selected top 300 coexpressors of each COQ query genes (first cut-off)-that intersect with at least 2 of these query genes (second cut-off). For legibility, flavonoid biosynthetic genes are now identified by numbers and are highlighted. We have also added a paragraph to the figure legend to provide more details about how the co-expressors were mined and how the network was reconstructed. Last, we have included in Supplemental dataset 1, the lists of aggregated co-expressors (i.e. *COQ1* vs. *COQ8* vs. *COQ9*; *COQ1* vs. *COQ8*; *COQ1* vs. *COQ9*; *COQ8* vs. *COQ9*), so other investigators can re-construct and mine the network themselves if they wish to do so. Note that the hierarchical cluster of Figure 1C is a different in silico experiment. Its aim is not to mine for interacting genes or make the claim that flavonoid biosynthetic genes co-express with ubiquinone biosynthetic genes. Instead, this hierarchical clustering compares the relative distances between the entire co-expression profiles (>22,200 loci) of known ubiquinone and flavonoid biosynthetic genes (the principle is similar in spirit to what one would use for a phylogenetic reconstruction or metabolite clustering using a distance method). There is no cut-off here.

The last discussion section of "A Paradigm Shift for..." is important for Breakthrough Reports and can be reworked to better highlight the significance of the study. I agree that the novel function of kaempferol in UQ biosynthesis is very interesting, but the authors could expand more in the context of the evolution of plant primary and secondary metabolic pathways. A general dogma is that primary metabolites serve as precursors to synthesize a variety of secondary metabolites, due to step-wise evolution of core to specialized pathways. This study revealed that a secondary metabolite (kaempferol) can now serve as a precursor of a primary metabolite (UQ) in plants and possibly in animals. If the authors agree, this point could be highlighted also in the abstract (e.g. perhaps, within the sentence of Line 41-43).

We agree indeed. We have re-worked the abstract and the discussion to better reflect the novelty of our findings. We have also expanded the discussion on the topic of the evolutionary features of the metabolic connection between flavonoid and ubiquinone biosynthesis.

Minor comments: Figure 2D -I wonder if the authors have conducted feeding of quercetin as a comparison.

No, we have not, since loss of function of *f3'h* (kaempferol --> quercetin) does not impact ubiquinone biosynthesis.

For general readers, Figure 2 will be difficult to understand without looking up Supplemental Figure 2. It may be helpful to include at least a simplified diagram of the flavonoid pathway (may be without structures) within Figure 2.

We agree that without supplemental Figure 2 as a companion panel 2D might be confusing for the nonexpert. We have therefore included a simplified biosynthetic branch that represents the metabolic connections between naringenin, dihydrokaempferol and kaempferol in panel 2D.

Figure 2D -It may make more sense to indicate stats between WT and *f3h* within respective treatment to see if the significantly reduced UQ content in *f3h* is or isn't recovered by various metabolite feedings.

We agree. The statistics and figure legend have been modified accordingly.

Line 253 -"Plants Posses..." to "Plants Possess...."

This has been corrected. Thank you for pointing this out.

Line 284 -Alternative explanation could be that dihydrokaempferol may be channeled between F3H and FLS and could not be easily taken up to the pathway.

Absolutely. We have added a sentence to mention this possibility. Actually, the two scenarios (C-2 C-3 double bond formation and channeling of dihydrokaempferol) are not mutually exclusive. C-2 C-3 double bond formation, however, is an absolute pre-requisite for C-ring cleavage.

Line 315 -Should it be "activity", instead of "Km"?

Km (a concentration) is correct. In other words, even if in vivo the cleavage rate of kaempferol were as low as that measured in vitro for a concentration of kaempferol representing only 1% of Km, the total pool of free kaempferol in the cell would be cleaved in a few minutes.

Line 332 -Insert the phrase [], "...under strong selection pressure [: they are maintained] even in ...."?

We apologize: we do not understand exactly the change in sentence construction that the reviewer is suggesting.

Line 334-336 -I don't think this sentence is fully integrated into the paragraph. Line 336 -A new paragraph can be started.

We have started a new paragraph and modified the title of this section, so as to include the nutritional relevance of flavonols.

Line 376 -Please cite a reference of a molar extinction coefficient of kaempferol.

The reference has been added.

TPC2018-00688-BR1 2nd Editorial decision – *acceptance pending* **Oct. 18, 2018**

We are pleased to inform you that your paper entitled "The Peroxidative Cleavage of Kaempferol Contributes to the Biosynthesis of the Benzenoid Moiety of Ubiquinone in Plants" has been accepted for publication in *The Plant Cell*, pending a final minor editorial review by journal staff.

Final acceptance from Science Editor **Nov. 13, 2018**