Inferring Roles in Defense from Metabolic Allocation of Rice Diterpenoids

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Review timeline:

TPC2015-01059-RA	Submission received:	Dec. 22, 2015
	1st Decision:	Jan. 29, 2016 *manuscript declined*
TPC2018-00205-RA	Submission received:	March 8, 2018
	Final acceptance:	March 23, 2018
	Advance publication:	April 24, 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.)

TPC2015-01059-RA 1st Editorial decision – *declined* January 29, 2016

Thank you for choosing to send your manuscript entitled "Inferring Roles in Defense from Metabolic Partitioning of Rice Diterpenoids" for consideration at The Plant Cell. Your submission has been evaluated by members of the editorial board as well as expert reviewers in your field, and we regret to inform you that we are not able to recommend publication of this manuscript. We have not made this decision lightly. We have had input from multiple scientists, and have solicited post-review comments as well. Our present policy is to offer streamlined decisions and to not advise on the direction of the work by requesting extensive modifications or substantial additional experiments.

Reuben, this was a difficult decision for me. I would like to have asked for a revision as the reviewers were generally supportive, with some caveats that could be addressed. The sole reason I can not is the fact that one of the main lines of data in the paper, that for the *cps4* mutant, is based on a single mutant allele. Plant Cell's policy is that when substantive data that is a central component of the manuscript is based on a mutant, as is the case here, more than one line of genetic evidence must support it, i.e. there must be a second allele with similar/supportive data or the single allele must be complemented (as you did with *cps2*) and the complementing phenotype provide supportive/confirmatory data (reversion of biochemical/physiological phenotype, etc). As it would take substantial effort to generate a complemented *cps4* mutant line or alternatively identify a second knockout mutation, and as we are no longer allowed the option of reject with encouragement to resubmit (to avoid endless rounds of submission, review and resubmission) the only option left to me is decline. If you had a *cps4* complementing line analogous to the *cps2* complementing line or a second mutant allele to support the data, I would be writing a different decision letter. If you are developing such a line and it will be available in a reasonable time frame you could rebut this decision. We are willing to hear thoughtful rebuttals after you have had some time to digest these comments.

Regardless of the ultimate outcome, there are comments from the reviewers that should be taken into account in any future submission of the work, to Plant Cell or another journal.

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

[Reviewer comments shown below along with author responses]

TPC2016-00205-RA Submission received March 8, 2018

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

Reviewer #1:

The manuscript "Inferring Roles in Defense from Metabolic Partitioning of Rice Diterpenoids" Examines the relative roles of rice CPS2 and CPS4 in producing diterpenoids for plant defense. The authors show that the diterpenoids produced from ent-CPP by CSP2 are important for defense against both fungal and bacterial pathogens of rice, while diterpenoids produced from syn-CPP by CPS4 may play a role in non-host resistance to certain fungal pathogens. Furthermore the authors show that loss of CPS4 leads to increased levels of certain ent-CPP diterpenoids presumably due to increased access to their shared precursor GGPP.

Importance of findings:
The demonstration that CPS2 is important for the defense of rice against fungal and bacterial pathogens using gene knock-out and overexpression approaches provides strong evidence for its function in disease resistance that can only be inferred from in vitro studies. Furthermore, the inferred role for *CPS4* in non-host resistance is a new finding for this gene that is of general interest to this field of research. This work also is a good demonstration of the metabolic links and balances that exist between pathways that is relevant to the wider community.

Quality of experiments
In general the experiments appear to be of high standard although a little more detail in the methods would not go amiss, especially regarding disease phenotype scoring.

Points In favor
This is a clear, generally well written study with solid conclusions. The scope is sufficient and the discussion well thought out.

Points detracting
A lot of weight regarding the function of the diterpenoids in defense in this paper is assigned based on visible disease symptom development and while this often correlates with the level of pathogen infection the conclusions would have been more robust with some measure of pathogen levels. i.e. bacterial growth or fungal DNA levels.

Point 1. Add to supplemental Data growth curves for OS-CPS2OE plants as it is mentioned twice in the manuscript that there is no difference, these data should be included.

RESPONSE: These data have been added as requested.

Point 2. On page 9 the authors state that *Os-cps2* and *Os-cps4* plants accumulate increased levels of casbene compared to their wild type parents. This data is not clear from the figures. The authors need to add numerical data for this statement and appropriate statistics.

RESPONSE: The data for ent-casbene have been added to the manuscript (new Fig. 6C), as requested.

Reviewer #2:

The in vivo functional characterisation of enzymes of the terpenoid specialised metabolism and identification of facets of their physiological role remains a major scientific challenge, even in model plants. In rice, the biochemistry of diterpene metabolism is very well understood, so are the bioactivities of the individual compounds on relevant pathogens.
Lu and co-authors report modulation of the diterpene profile in rice in an identified null mutant of one of the two class II ent-copalyl diphosphate synthases and corresponding lines over-expressing the gene. A previously identified null allele for the second, and functionally distinct class II syn-copalyl diphosphate synthase is included in the study which focuses on the impact of changed metabolite pattern on rice-pathogen interactions.

As the two diTPS represent the distinct entry (and control) points defining the stereochemistry of the two dominating

classes of diterpenes in rice, this approach is ideally suited to dissect the impact of suppressed and overproduced ent-CPP and suppressed syn-CPP derived diterpenes on the plant-pathogen interaction.
Altered accumulation of the ent-type diterpenes was shown to correlate with the phenotype, i.e. increased resistance or susceptibility, linking the molecules with a function in defence. Interpretation of findings of the syn-CPP suppressed line was less straightforward. Yet, the authors find a reduced resistance to non-host pathogens and also propose that the observed resistance against a rice pathogen is related to metabolic re-routing of the shared precursor of the pathway into ent-type diterpenes.

The findings appear scientifically sound and are potentially of interest for a broad audience if better presented.

My main critique is a lack of concise and sharp writing. The flow and logical connections are at times very difficult to follow. Especially in the abstract and introduction over-generalisations and -simplification disappoint in an area with a wealth of highly specific knowledge. In the text, where additional context is provided there are several instances, where the relevance is questionable or confusing. Further, typos and wordings such as 'more generalised' or 'more specialised' diterpenes give the impression that this text has not received appropriate attention. Perhaps symptomatic is that the reader does not learn about the approach of over-expression of the ent-CPPS until the third chapter of the results, which makes it difficult to understand why this study is about metabolite partitioning. The results sections and the discussion are also not well balanced, with the discussion covering about as much as a single chapter of results.

Point 1. There is very little information in the summary beyond what is given in the title. Possibly specify 'physiological function'.

RESPONSE: Revised as requested.

Point 2. Plant bioactive compounds that respond to pathogen attack are not generally identified based on in vitro activity.

RESPONSE: Keeping in mind that the emphasis here has shifted to use of metabolic allocation to suggest non-obvious roles (particularly here in non-host disease resistance), we are simply noting that, presumably reflecting the difficulty in generating genetic evidence in plants beyond *Arabidopsis thaliana*, it seems difficult to find phytoalexins that do not include in vitro antibiotic activity as a critical piece of evidence for their relevance to defense. Please note that this includes the use of extracts, which are not pure compounds, but have been extensively used (not least in rice) for purification and identification of specific compounds such as the diterpenoids investigated here. Given the traditional focus on known pathogens, this then leads to the noted issue of missing the roles that phytoalexins might play in non-host disease resistance.

Point 3. As the Oscps2 line is a single line from a transposon population, a clear link between the mutation and the phenotype is important. An observed phenotype can, for example, be result of background mutations. This could typically be achieved with a second allele, or complementation. The observed phenotype could also be result of reduced overall fitness. However, the later following paragraph about the overexpression alleviates this issue somewhat.

RESPONSE: We now include RNAi lines for both Os-CPS2 and Os-CPS4.

Reviewer #3:

In this report, the respective contribution of rice labdanoid diterpenoids to resistance against pathogens is evaluated. Rice labdanoids from secondary metabolism can originate from two distinct precursors, either ent-CPP (common to gibberellins) or syn-CPP. These precursors are produced by two distinct copalyl diphosphate synthase enzymes, OsCPS2 for ent-CPP and OsCPS4 for Syn-CPP. By analyzing knock-out plants for each of these genes, the respective contribution of the groups of derived diterpenoids to pathogen resistance could be inferred. The major findings are that the ent-CPP derived diterpenoids contribute to resistance to rice pathogens like *Magnaporthe oryzae* or *Xanthomonas oryzae* pv. oryzae, whereas those from syn-CPP do not. Interestingly, in oscps4 plants, the levels of ent-CPP derived labdanoids were higher, coinciding with an increased resistance against Xoo. This effect was reproduced by over-expressing OsCPS2. Another interesting finding is the role of OsCPS4 derived diterpenoids

in non-host resistance against other strains or species of Magnaporthe.
The manuscript is well written with a clear and logical progression. The figures illustrate well the points made in the text. The results are of broad interest, not only to the terpenoid biosynthesis community, but also to the plant pathogen community. Some additional experiments suggested below (in order of importance) would however significantly strengthen the manuscript.

Point 1. It is always assumed by the authors that the active compounds are the end-products of the pathway, e.g. the oryzalides (for OsCPS2 derived) or the momilactones (OsCPS4 derived). Yet they only measure the diterpene precursors, not the supposedly active products. Measurements and quantification of these compounds (by LC-MS) would add significant support to these hypotheses and provide a more detailed and informative picture and the contribution of these diterpenoids to disease resistance.

RESPONSE: We have carried out extensive studies to develop a comprehensive method to analyze the diterpenoid end-products, at least where known, as now described in the revised manuscript.

Point 2. Since only one mutant of *OsCPS2* is characterized and because it is generated by Agrobacterium transformation, which is known to cause multiple insertions, either a complementation or at least a demonstration that there is only one insertion in this line is required.

RESPONSE: As noted above, RNAi lines are now included to support the single knock-out line.

Point 3. Since OsCPS4-derived diterpenoids are involved in non-host resistance, one could, perhaps naively, infer that these are more ancestral than the OsCPS2-derived ones. What does the phylogenetic analysis of CPS and KSL sequences of rice say? Is there any trend that can be seen there in correlation with the function (contribution to resistance against non-host vs host pathogens)?

RESPONSE: This is an interesting hypothesis. However, our previous phylogenetic analysis of CPSs from across the cereal plant family does not support earlier evolution of Os-CPS4 versus Os-CPS2 (actually the opposite is suggested). The relationship among the KSLs is difficult to discern, in part due to the substrate promiscuity we have previously reported, which makes it difficult to determine how these pair with the upstream CPSs that produce different stereoisomers (i.e., such as Os-CPS2 and Os-CPS4).

TPC2018-00205-RAR 1st Editorial decision – *acceptance pending* **March 23, 2018**

I have personally reviewed your new submission in detail, the prior reviewer's comments, your responses and paid close attention to the revisions to text you have made in the current submission. Recall that reviewers were overall positive of the last submission, requested some revisions for clarity and to engage a more general audience and that the primary reason for my decision to not accept the prior submission was the lack of a second allele for one locus on which you had done a large number of experiments and drawn conclusions. You've now provided additional RNAi lines that support the previous single allele conclusions and based on this, and my own review of the manuscript and your appropriately addressing the bulk of prior reviewer's comments I'm pleased to inform you that, in consultation with other TPC editors, I have determined that the manuscript is acceptable for publication and that it does not require additional reviewers. This is the first time I've done this and it reflects the overall excellent quality of the work in the prior submission, the continuation of that quality in this one and the fact that had an additional allele been available in the first submission (which supported biochemically and biologically with the single allele) that I would have accepted the prior paper pending revisions in response to reviewers (which you have now done). Undoubtedly the Science Editor will suggest changes in regard to content presentation etc., but scientifically the new manuscript is sound and something I look forward to seeing in press. I appreciate your taking the prior rejection in the way it was attended, addressing the issues in such a forthright way and deciding to resubmit the work to TPC for consideration again.

Final acceptance from Science Editor **April 23, 2018**