Discovery of UDP-Glycosyltransferases and BAHD-Acyltransferases Involved in the Biosynthesis of the Anti-Diabetic Plant Metabolite Montbretin A

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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.)

TPC2018-00334-RA 1st Editorial decision – *declined* March 19, 2018

All reviewers agreed the work was of very high technical quality and the conclusions made on the enzymatic activities of the two enzymes fully supported by the reported in vitro studies. They also recognized that characterization of a flavonoid UGT from the P clade is important. However, two reviewers independently concluded that while the study was excellent from an experimental/technique perspective, the characterization of the two UGTs did not meet the novelty expectation for a Plant Cell paper. My own reading of the manuscript led me to the same conclusion. At least two possibilities could allow the study to clear this bar. One would be in vivo demonstration that either of the UGTs is directly involved in the MbA pathway, as the current data is strongly suggestive, but not conclusive, in this regard. This would entail in vivo downregulation, transient or stable, of at least one of the two enzymes and demonstrating a corresponding impact on MbA synthesis, or intermediate accumulation etc. I recognize this is likely impractical as you are working in a non model system, and I am acutely aware of the inherent difficulties of such experiments in these organisms, though if there is potential in this arena it would be worthwhile. A second and perhaps more practical approach is if the acyl transferase of the pathway were identified and some limited characterization of it included. Two of the reviewers independently suggested this would easily push the paper over the novelty bar, with which I concur.

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[Reviewer comments shown below along with author responses]

TPC2018-00406-RA Submission received May 29, 2018

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

Reviewer #1:

The authors elucidated a biosynthetic framework and possible reaction route for the production of montbretin A, which is a highly important phytochemical having a potent inhibitor activity of the human pancreatic alpha-amylase. In this manuscript, the authors performed plenty of enzymatic assays and metabolite profiling as well as transcriptomic analysis and transformation in order to characterize two genes encoding flavonoid glycosyltransferase involved in the

montbretin A pathway. I believe that the obtained results in this manuscript are highly important, since the authors discovered novel functional genes via functional genomic approach using such non-model plants producing highly beneficial metabolites. Authors carefully performed experiments using a combination of different approaches, such as an enzyme activity assay using total protein and a recombinant assay with different sets of the substrates. Annotation and identification of detected compounds are properly performed. I have just some minor points below.

Point 1. Abstract: Describe the enzymatic function of UGT709G2 like "3-O-rhamnoside-2"-O-glycosyl" in the abstract.

RESPONSE: The additional descriptions would add to the length of the abstract, which is already at 250 words. We are therefore providing the full descriptive names of the two UGTs and the new ATs in the last paragraph of the Introduction.

Point 2. Figure 3C: Peak 3 might be putative myricetin-3Rha-Glc-Glc, which is also a possible intermediate of montbretin A?

RESPONSE: We reviewed our data for this comment. Figure 3C shows the extracted ion-chromatogram of m/z 625. Therefore, peak 3 cannot be annotated as MRGG, which would be m/z 787. MRGG would also not be expected to fragment into m/z 625. In the new Supplemental Figure S8, we show that m/z 787 produced in assays with corm protein extracts is MRG with an additional glucose attached to the flavonol ring. We did not observe the formation of MRGG as an intermediate in MbA biosynthesis. This is now stated in the text.

Reviewer #2:

This is a very nice piece of work identifying two UGTs involved in the biosynthesis of a complex acylated flavonoid glycoside with important biological activity for the treatment of type 2 diabetes. The biochemical analysis of the products formed both in vivo and as a result of enzyme catalysis is very solid, and there is generally little to criticize. It is a shame, however, that the authors were not able to also use gene co-expression analysis to identify the acyltransferase that would complete the pathway to mini-MbA. If they have now done this, I urge them to include the data in this manuscript. Other than this, I have only minor comments:

Point 1. Line 231. Is it likely that UGTs are NOT involved in the conversion of myricetin to MR and MRG.

RESPONSE: We have reworded the sentence, which now reads "To verify the conversion of myricetin to MR and MRG as the first two steps in MbA biosynthesis and the involvement of UGTs in MR and MRG formation ...".

Point 2. Line 263. Although probably correct, the way in which the glycosides are described is perhaps confusing to the uninitiated. Here myricetin 3-O-rhamnosyl glucoside describes a molecule in which the glucose is attached to the flavonol, but this seems counterintuitive based on the description. The MGR abbreviation is clearer, and this type of descriptor should always be used in the manuscript (e.g. line 275).

RESPONSE: We agree that the conventional descriptors are counterintuitive, which is why we added the abbreviations. We followed the reviewer's suggestion and included abbreviations for all the molecules.

Point 3. In some ways, the differential transcriptome analysis did not work well, resulting in 70 candidates that were only finally resolved based on phylogeny, not expression pattern. It would have been helpful if other tissues had been included in the reference transcriptome (although this obviously would not affect the final outcome).

RESPONSE: We agree. The differential transcriptome analysis reduced the number of candidate genes from 159 to 70 for UGTs, which indeed was still a large set of candidates that was reduced further by phylogeny. As described in the manuscript, for the discovery for UGT709G2 and now CcAT1 and CcAT2, we did indeed use additional expression analysis in different montbretia organs (flower, leaf, stem, stolon, corm) to look for co-expressed genes using the expression pattern of UGT77B2 as bait.

Point 4. The expression studies in tobacco highlight one potential problem with using such a plant platform for the synthesis of compounds such as MbA, namely the promiscuous activity of the UGTs for other plant-derived acceptor molecules, necessitating potentially costly separation methods. Although this promiscuity is mentioned, it might be helpful to compare plant-based systems with microbial platforms that would be cleaner- there are several papers reporting high glycosylation efficiencies for flavonoids fed to engineered *E. coli*, for example. Microbial systems are mentioned, but not compared.

RESPONSE: The reviewer is correct. We have now extended the discussion on the potential of plant and microbial production systems.

Reviewer #3:

The acylated flavonol glycoside montbretin A (MbA) provides a promising drug for the treatment of type-2 diabetes. As was already determined in 1988, MbA is found in the corms of montbretia (*Crocosmia* x *crocosmiiflora*). Identifying the pathway for the biosynthesis of montbretin to engineer the formation of these compounds is thus of importance.

This manuscript describes the identification of two glycosyltransferases (GTs) that participate in the initial steps of the formation of MBa from the flavonol myricetin. The authors convincingly show that UGT77B2 and UGT709G2, identified from RNA-Seq experiments on corms, catalyze consecutive glycosylations of myricetin to produce myricetin 3-O-rhamnoside (MR) and myricetin 3-O-glucosyl rhamnoside (MRG). Demonstrating that these enzymes are the ones responsible for the formation of MRG in vivo in montbretia would require mutants. Nevertheless, the studies provide a significant step forwards towards the possibility to engineer the pathway.

Point 1. Perhaps more disappointing is that the acyl-transferase necessary for the formation of mini-MbA, which is also a potent pancreatic α-amylase inhibitor, was not identified in this study.

RESPONSE: We also very much appreciate the positive comments of this Reviewer. Based on this Reviewer's comment, and following the suggestion of Reviewer #2 and the Editor's recommendation, we added the results from new work on the acyltransferase.

Point 2. Lane 147: It is not completely accurate that "... less is known about the glycosylation of flavonols". In Arabidopsis alone, the following UGTs involved in flavonol glycosylation have been identified: UGT78D1, UGT73C6, UGT78D2, UGT89C1, UGT78D3, UGT79B6, and UGT79B1. It would have been interesting to compare the participation of these enzymes in flavonol glycosylation with those identified in this study, particularly given that the enzymes can also use kaempferol.

RESPONSE: We agree that the original wording was not accurate and deleted the statement. We also included all of the mentioned UGTs into the phylogeny in the new Figure 4. In the text, we compare montbretia UGT77B2 with the only other identified 3-*O*-rhamnosyltransferase, UGT78D1 from *Arabidopsis*. We added a comparison of montbretia UGT709G2 with the flavonol 3-*O*-glucoside:1,2-*O*-glucosyltransferase, UGT79B6 from *Arabidopsis*.

Point 3. The profiling of presumed pathway intermediates in corms of different ages can provide evidence for the possible pathway steps. However, because the analysis is based on targeted profiling with specific m/z values, it is possible to miss intermediates that are formed by unusual steps.

RESPONSE: We fully agree that intermediates that may be the result of unusual reactions could not be excluded a priori. As the metabolite levels of intermediates were quite low, we relied on targeted metabolite profiling and predicted m/z for the most likely intermediates. This approach validated predicted intermediates.

Point 4. The authors don't mention the formation of montbretin B, which contains a coumaroyl rather than the caffeoyl group present in MbA. While perhaps not very likely, the studies don't formally demonstrate that the identified enzymes participate in MbA formation and not in MbB formation. At the very least, the existence of MbB needs to be acknowledged and this possibility discussed.

RESPONSE: We appreciate this comment. We have now included text in the Discussion on MbB and MbC, which have a coumaroyl or feruloyl moiety, respectively, instead of the caffeoyl moiety in MbA. We discuss that the UGTs and the newly added CcACT1 and CcACT2 may indeed contribute to the biosynthesis of different montbretins and that the specific product may be affected by substrate availability.

Point 5. It is interesting that MR was detected in corm extracts (and hence deduced as a pathway intermediate), the simultaneous expression of UGT709G2 and UGT77B2 in *N. benthamiana* resulted in complete conversion into MRG.

RESPONSE: This result may be due to the different levels at which montbretia corms and transformed *N. benthamiana* leaves produced these intermediates. The amounts of MR may have been below the detection limit in *N. benthamiana* leaf disc assays. Although we did not detect MR in *N. benthamiana* leaf disc assays when both

UGTs were expressed and myricetin was added, leaf extracts of *N. benthamiana* contained trace amounts of KR when UGT77B2 and UGT709G2 were expressed without feeding of myricetin. Higher levels of KR were present when CcAT1/2 were expressed in addition, suggesting that substrate availability as well as flux and enzyme expression are variable in this system.

Point 6. The enzymatic assays using the corm protein extracts, sugar donors and myricetin provided the authors with a possible set of candidate enzymatic activities to pursue. However, as the formation of myricetin xylosyl rhamnoside indicates, the results can be somehow misleading.

RESPONSE: The reviewer is correct. The assays with corm extracts informed our prediction of possible reaction in the formation of MbA, but we were cautious not to dismiss that in the absence of strict substrate specificity of UGTs, the results could have been misleading. In the specific example of MRX, we found 10-fold more MRG to be formed in the assays, and we also mention that MRX is not likely a possible intermediate in MbA formation.

Point 7. It is unclear how the various UGTs from other species were selected for inclusion in Fig. 5. For example, there are some maize UGTs that have been shown to be involved in glycosylating flavonoids that are clearly absent.

RESPONSE: To associate montbretia UGTs with previously described UGT clades, we selected *Arabidopsis* and maize UGTs that represent clades A-P. In addition, we selected characterized UGTs that catalyze *O*-glycosylations and chain elongations. This is now mentioned in the text. Following this Reviewer's comment and the suggestion of Reviewer #1, we included additional UGTs known to glycosylate flavonoids in the new Figure 4. We added UGT73C6, UGT78D2, UGT89C1, UGT79B6 from *Arabidopsis*; Gt3GT from gentian, Mt3pGT from *Medicago*, and Zm5,3GT and ZmF7O3GT from maize.

TPC2018-00406-RA 1st Editorial decision – *acceptance pending* **June 14, 2018**

We are pleased to inform you that your paper entitled "Discovery of UDP-Glycosyltransferases and BAHD-Acyltransferases in the Biosynthesis of the Anti-Diabetic Plant Metabolite Montbretin A" 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.

Reviewer #2 (Comments for the Author):

My major concern with the previous submission was the partial completion of the pathway. The new data on the acyltransferases now make this a very strong paper. It was also good to see the expression profiling having a more direct role in the identification of the latter enzymes. The authors should be congratulated on solving the biosynthesis of these important metabolites in such a thorough and well-documented way.

Reviewer #3 (Comments for the Author):

The authors have addressed pretty much all the concerns previously raised, and the inclusion of both acetyltransferases rounds-up a very nice story.

Final acceptance from Science Editor **June 27, 2018**