A Regulatory Hierarchy of the Arabidopsis Branched-chain Amino Acid Metabolic Network

Anqi Xing and Robert L. Last

Plant Cell. Advance Publication May 18, 2017; doi: 10.1105/tpc.17.00186

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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.)

TPC2017-00186-RA 1st Editorial decision – revision requested March 30, 2017

All reviewers recognized the importance of the results described in this study; however, each requested additional discussion of particular points raised in the manuscript. Reviewer 3 in particular raised three important aspects that require further discussion, and had good editorial suggestions for streamlining the Results section for improved clarity. Reviewer 3 also pointed out that data in Fig 7, 11, and Supplemental Table 6 require different statistical treatment than a t-test. Consider an improved pathway figure per reviewer 2 and additional clarification to figures and text suggested by reviewer 3.

The authors might also consider some commentary on how these results could interplay with other forms of regulation of these enzymes and how these effects may permeate throughout the metabolic network. While the focus of the manuscript is a strength, it would help the general reader to foresee the integrative implications of these results beyond BCAA's better.

Reviewer comments:

[Reviewer comments shown below along with author responses]

TPC2017-00186-RAR1 1st Revision received April 12, 2017

Reviewer comments and author responses:

Reviewer #1:

The manuscript by Xing and Last presents a comprehensive and convincing characterisation of the regulatory hierarchy of the branched-chain amino acid metabolic network. The study includes identification and comparative analysis of mutants as well as detailed enzymatic characterization of the proteins encoded. The experiments are clearly carefully carried out and well controlled they are also sufficient in order to support the conclusions that they draw. I have only two suggestions that I feel would improve the Discussion although in truth I regard as discretionary.

Point 1. The authors cite the work of Curien. It may be useful to explain how the data presented here could be built into current mathematical models of the amino acid network.
RESPONSE: We added the text, “and could contribute to future testing and refinement of mathematical models for this network” to page 17 of the manuscript.

Point 2. Whilst it is implicit that the authors regard BCAA biosynthesis to be all important in mediating homeostasis, it may be work discussing this in more detail. This is no rare thing in plant amino acid homeostasis given the presence of many such feedback loops, some, but not all of which are conserved also in microbes etc.

RESPONSE: We sincerely hope that we did not oversell the importance of allostery, and tried to maintain balance with the mention of catabolism in seed aa homeostasis (several mentions in the Introduction and the last paragraph of the Discussion), which we have studied over the past decade.

Reviewer #2:

In this work, the authors have presented a thorough study of in vitro regulation of the BCAA in seedling and seeds. This work provides a much needed in planta context for the extensive knowledge that has been gathered from in vitro experiments. The authors have focused on the three allosteric committing enzymes: threonine deaminase, acetoxyhydroxyacid synthase, and isopropylmalate synthase 1. Using extensive forward genetics, enzyme activity measurements, and reverse genetics, the authors demonstrated the dominance of threonine deaminase in controlling Ile accumulation and showed that this accumulation is largely independent of the two other enzymes, AHAS and IPMS. This is a surprising and interesting finding considering the fact that the three BCAAs share three enzymes in their biosynthetic pathway, which are downstream of threonine deaminase. However, the evidence provided is convincing and suggests a separate role for Ile accumulation in the tissues studied. The study also nicely demonstrates that AHAS and IPMS are mostly responsible for the Val/Leu accumulation and partition. This study proposed a regulatory hierarchy model in which TD, AHAS, and IPMS control BCAA levels in leaves and seeds, and that paralogous isoforms contribute unequally to regulation. Validating this model in both seeds and leaves is essential, since it was demonstrated that, in some cases, metabolic pathways regulation differs substantially between these tissues. Overall, the authors have provided extensive evidence to support their proposed model, and by that increase our understanding of the regulation of free BCAA in plants.

Point 1. Although I agree with the authors that understanding the metabolic pathway is the key for future biofortification, there is a need for clarification about the role of FAA vs. protein-bound amino acids and to point out the important but somewhat limited potential of FAA in biofortification.

RESPONSE: We agree that manipulation of both protein quality (for instance through storage protein modification) and free amino acids provide opportunities; this manuscript is focused on pathway regulation.

Point 2. The authors might want to clarify if there were any deleterious effects on germination or overall growth that occurred, since that is the case in some mutants that accumulated other amino acids. It would also help to discuss this issue in the context of biofortification.

RESPONSE: We did not see consistent or strong impacts on seed germination or overall growth. We added a statement about this general topic to the second paragraph of the Discussion.

Reviewer #3:

Branch chain amino acids (BCAAs) are essential nutrients in the human diet. Multiple feedback regulations of key enzymes involved in BCAA biosynthesis have been reported. However, little is known about the in vivo functions of such enzyme regulation in the overall homeostasis of BCAA.

The study by Xing and Last conducted forward genetic screenings of EMS-mutagenized Arabidopsis plants that are resistant to toxic analogs of isoleucine (OMT) and leucine (TFL). Mutations were found in amino acid-binding regulatory domains of threonine deaminase (OMR1) for OMT resistance, and of acetohydroxy acid synthase small
subunit 1 (AHASS1) and isopropylmalate synthase 1 (IPMS1) for TFL resistance. Biochemical characterization of recombinant enzymes showed that the mutated enzymes of OMR1 were more resistant to Ile-mediated inhibition, whereas those of AHASS1 were more resistant to Val and to some level Ile, and that of IPMS1 was slightly resistant to Ile. BCAA metabolite analysis of these mutant leaves further showed that the mor1D mutation led to high levels of Ile (and some Val and Leu), ipms1D increased Leu and decreased Val, whereas ahass1D increased both Val and Leu (and some Ile). Heterozygous F1 double mutants essentially had additive effects. These feedback-resistant mutations also had similar effects on seed BCAA composition and levels, though to a lesser degree. To further investigate the contribution of different isoenzymes of AHASS and IPMS, T-DNA knockout or knockdown mutants were isolated and further characterized. The loss of IPMS1 and AHASS2 had more pronounced effects on BCAA levels than IPMS2 and AHASS1, respectively.

The study combined forward and reverse genetics, and biochemical enzyme characterization to demonstrate how feedback regulations of committed enzymes are coordinated to control BCAA homeostasis in plants. Given the importance of BCAA as essential human nutrients, the knowledge and mutations obtained in this study can be translated to enhance nutritional quality in crops through transgenic, genome editing, and breeding approaches. Thus, the study likely is of interest to a broad audience in the field of plant biochemistry and biotechnology.

Point 1. The effect of the G606E mutation on the Leu sensitivity of IPMS1 was quite minor (Fig 5D) relative to those of OMR1 and AHASS1 (Figs 3D and 4C). Also, the G606 residue was not highly conserved and located away from the predicted Leu binding pocket. Thus, I am not fully confident about the authors’ conclusion for strong links among the G606E substitution, in vitro Leu sensitivity, and in vivo TFL-resistance. Thus, I would like to see more careful evaluation of alternative possibilities, such as the effects of the mutation on i) other enzymatic properties through kinetic analysis for IPMS1 wild type and mutant (which was provided for OMR1 and AHASS1 but lacking for IPMS1), ii) possible activation or antagonization by Val, as well as iii) IPMS1 and IPMS2 expression levels in the ipms1-1D mutant as compared to wild type.

RESPONSE: Given the length and scope of this manuscript, we plan to explore how changes in kinetic properties and expression of this and other ‘regulatory’ enzymes influence amino acid homeostasis in Arabidopsis BCAAs in future work.

Point 2. The effects of feedback-resistant mutations and T-DNA insertions on overall plant growth and development are not presented. Metabolite data are normalized by mg fresh weight and it is important to know if overall plant weight was different between genotypes. Also, given that altered amino acid contents are known to often affect plant growth and development, the effects of each metabolic perturbation to overall plant growth is critical information, especially in terms of future application to plant metabolic engineering. If growth was compromised in some lines, it may explain some of the expected results, such as increases in Val and Leu in omr1-11D (Fig 6B) and Leu in the ipms2 ipms1-1D double mutant (Fig 8D).

RESPONSE: See sentence added to the next to last paragraph of the Discussion.

Point 3. IPMS1 consistently had major effects on carbon partitioning between Val and Leu when downregulated by T-DNA insertion and also when mutated to make it resistant to feedback inhibition by Leu. On the other hand, AHASS2 had major effects on BCAA homeostasis when downregulated by T-DNA insertion, whereas only AHASS1 was isolated from the EMS mutagenesis screening. Also, the ahass2-1 mutant resulted in increased accumulation of Val. Furthermore, Ile could rescue the inhibitory effects of Val+Leu treatment in wild-type seedlings, which presumably inhibits AHASS2 in vivo. The basis of these observations is not well explained. It may be difficult to address experimentally in this already data-rich manuscript, but I would like to see better discussion beyond simply saying, “AHASS2 has a role in maintaining BCAA homeostasis”. Is it possible that AHASS1 and AHASS2 interact in vivo and function together (e.g. having both AHASS1 and 2 in a single heterotetramer of AHAS enzyme complex)? Does Ile treatment antagonize AHAS inhibition by Val and Leu?

RESPONSE: We considered a number of hypotheses during manuscript preparation, but all would require experimentation. We hope that publication of these results will stimulate work on how AHASS regulates BCAA homeostasis in plants.
Point 4. Data that compare more than two groups (e.g. Fig 7, 11, and Supplemental Table 6) require a different statistical analysis other than t-test (e.g. ANOVA).

RESPONSE: We now include one-way ANOVA and Duncan’s multiple range test on the data in these figures/tables.

Point 5. To better present BCAA inhibition at a wide range of concentrations among different enzymes, the X-axis (effector concentrations) can be presented in log scale. This will also allow calculation of IC50 values, which can be used to discuss the effects of different mutations on feedback regulation.

RESPONSE: After discussion, and considering the relatively narrow range of the substrate concentration used, we prefer not to use the log scale. Hopefully we did not misunderstand the suggestion.

Point 6. The Results section was lengthy mainly due to the amount of data presented, but also partly because of excess discussion of each result. Some interpretations of results are necessary for transitions to the next experiments; however, some detailed discussion could be left for later in the Discussion session (indeed, many statements were repeated in both the Results and Discussion sections).

RESPONSE: We strongly agree that Results should not discuss and the Discussion should not only reiterate results, and we carefully reviewed the text to try to trim as suggested. We originally chose to use the ‘here is what we told you’ style of paragraph conclusion in the Results because of our experience that it is very easy to cause non-biochemists to disengage, and we did our best to make these ending sentences short in the initial writing. We are hard pressed to find sentences to delete and welcome suggestions. The Discussion is only five paragraphs long and we cannot find a way to trim enough text to make a difference. Any specific advice would be most welcome.

TPC2017-00186-RAR1 2nd Editorial decision – acceptance pending April 14, 2017

Thank you for your diligence in addressing the editorial and reviewer comments in such a thorough fashion. We are pleased to inform you that your paper entitled "A regulatory hierarchy of the Arabidopsis branched-chain amino acid metabolic network" 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 May 11, 2017