Review of: "A peroxisomal heterodimeric enzyme is involved in benzaldehyde synthesis in plants"

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Review of 'Peroxisomal heterodimeric enzyme is involved in benzaldehyde synthesis in plants'.

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Review

Not only has this article provided the missing catalytic piece for the biosynthesis of volatile benzenoids, but it also has corrected the previous concept that 3-hydroxy-3-phenylpropionyl-CoA is a precursor for the formation of benzaldehyde in petunia (same group by Boatright et al., 2004). The article by Huang and co-workers (2022) has addressed a novel NADPH-dependent reductase that is benzoyl-CoA-specific and functional when both α and β subunits are combined to synthesize benzaldehyde in peroxisomes. Biochemical, proteomic and genetic analyses agreed with the spatial, developmental and rhythmic expressions of the α-subunit that precede the peak of benzaldehyde emission.

The detection of this heterodimeric enzyme can provide an important biosynthetic brick for the biotechnological production of benzaldehyde or possibly its substituted derivatives (i.e. vanillin) for industrial use. However, the article by Huang and co-workers questions the exclusive role of β-oxidative pathway in the biosynthesis of benzoyl-CoA; the benzaldehyde synthase substrate.

First section in the results shows the biosynthetic ability of petunia corollas to accumulate benzoyl-CoA after feeding them with benzoic acid. Benzaldehyde synthase (benzoyl-CoA reductase) uses benzoyl-CoA to produce benzaldehyde (23.03%; Huang et al., 2022). A CoA ligase that provides benzoyl-CoA substrate for benzaldehyde synthase is necessary to accomplish this step. To justify the accumulation of benzoyl-CoA-dependent products (ex. benzaldehyde and benzylbenzoate) after feeding benzoic acid, the article claims for a side activity from other aromatic-CoA ligases referring to two citations: 1) Klempien et al. (2012) and 2) Srividya et al. (2020) and uses the term 'Cinnamate-CoA ligases like enzymes' to refer to the claimed activity. While analysing the recent results in relation to these citations a missing aspect is to be considered. Klempien et al. is more related than Srividya et al. article as the earlier is a research work by the same group and also used petunia system as does the current work. In 2012, Klempien and co-workers had characterized twoCoA ligases from petunia; *Petunia hybrida* cinnamate CoA-ligase (*Ph*CNL) is unable to activate benzoic acid, which excludes its role from any catalytic participation that ends with the formation of benzaldehyde after feeding benzoic acid. The second characterized enzyme in Klempien et al. work was 4-coumarate-CoA ligase 1 (*Ph*4CL1) whose affinity to benzoic acid is 60 times less than its affinity to cinnamic acid. Accordingly, the catalytic efficiency of *Ph*4CL1 to benzoic

acid is 0.000124 that with cinnamic acid. Noteworthy, no localization experiments were done to *Ph*4CL1, but the protein lacks the peroxisomal targeting signal and is predicted to localize in the cytosol (Klempien et al., 2012).

The second article that is cited by Huang and co-workers is Srividya et al. (2020), which used a phylogenetic distant *Taxus media* to isolate and characterize 13 gymnosperm acyl-activating enzymes (AAEs). Four *Tm*AAEs (*Tm*AAE4, *Tm*AAE5, *Tm*AAE13, and *Tm*AAE15) were able to activate benzoic acid. *Tm*AAE4 was the most likely benzoate-CoA ligase (BZL) due to its unique preference for benzoic acid. A similar catalytic function has not yet been detected in petunia and the reported ability of *Ph*4CL1 to activate benzoic acid is still dramatically weaker i.e. the affinity of *Tm*AAE4 to benzoic acid (K_m=172) µM is 52 times higher when compared to *Ph*4CL1 (K_m = 9008 µM).

Previously, it was hypothesized that benzoyl-CoA is synthesized via 3-ketoacyl-CoA thiolase 1 (KAT1) integrated within the β-oxidative pathway in petunia (Van Moerkercke et al., 2009). The product of KAT1 is benzoyl-CoA and should be the substrate of benzaldehyde synthase discovered recently by Huang et al. (2022). The reduced thiolase activity in the transgenic lines with suppressed *Ph*KAT1 expression (ir-*PhKAT1*) was not tested using the correct 3-oxo-3-phenylpropionyl-CoA to produce benzoyl-CoA, but a general acetoacetyl-CoA substrate was used instead. At the metabolite level, Van Moerkercke and co-workers (2009) had analysed these transgenic lines that showed reduction in phenylethylbenzoate, and benzylbenzoate by 3.2 and 3.5 folds, respectively. In ir-*PhKAT1* lines, Van Moerkercke et al. (2009) showed a reduced emission of benzaldehyde by 2 folds only beside a surprising reduction in the level of methylbenzoate (by 2.7 folds) that is not dependent on benzoyl-CoA. The recent work by Huang et al. (2022) showed that the reduction in the expression of petunia benzaldehyde synthase has no effect on the level of methylbenzoate, which is expected but still opposite to the effect of the preceding ir-*PhKAT1* on the reduced level of the same scent product. Noteworthy, the work by Van Moerkercke et al. is cited and KAT1 and is integrated in the proposed pathway within Figure 1 of Huang et al. (2022).

A closer view at the values listed in Table 1 of the recent article by Huang and co-workers shows that the amount of labeled benzaldehyde after feeding benzoic acid is very comparable to that after feeding labeled L-phenylalanine. Feeding benzoic acid is followed by its direct activation to benzoyl-CoA (i.e. BZL activity), which is reduced to benzaldehyde by benzaldehyde synthase. On the other hand, the fed L-phenylalanine might be pooled into the β-oxidative pathway to produce 3-oxo-3-phenylpropionyl-CoA that is converted to benzoyl-CoA by KAT1 or it might be metabolized to benzoic acid that is converted to benzoyl-CoA by an un-defined BZL. In both cases the underlying biosynthetic sequences after feeding seems to be contributed comparably to form benzaldehyde (Table 1 of Huang et al., 2022). Benzaldehyde (benzoyl-CoA product) is reduced further to benzylalcohol, which reacts with benzoyl-CoA in the presence of benzoyl-CoA:benzyl alcohol/2-phenylethanol benzoyltransferase (BPBT) to produce benzylbenzoate. This shows that both substrates for the formation of benzylbenzoate depend exclusively on the benzoyl-CoA pool. Sufficient amount of benzoyl-CoA after feeding benzoic acid leads to a significantly higher amount of benzylbenzoate compared to that after feeding L-phenylalanine (Table 1 of Huang et al., 2022). It could be that L-phenylalanine is integrated in other phenylpropanoids pathways after its feeding, which explains the lower percentage of benzylbenzoate. However, these measurable amounts of benzaldehyde and benzylbenzoate products after feeding benzoic acid cannot be explained without the existence of an efficient BZL.

This is an experimental evidence that an aromatic-CoA ligase can contribute in non β-oxidative route to support a

comparable pool of benzoyl-CoA substrate for the catalytic functions of benzaldehyde synthase and BPBT. Information about petunia aromatic-CoA ligases enable efficient activation of benzoic acid is missing. Supporting biochemical data are basically needed for new petunia CoA ligases to safely mimic benzoate activation scenario in gymnosperm (Srividya et al., 2020). Moreover, the role of *Ph*4CL1 in benzenoid formation had been excluded in 2012 by the same group when Klempien and co-workers confirmed that RNAi of petunia 4CL1 had no effect on the emission of benzenoids (as shown in Suppl. Fig. 3 of Klempien et al. 2012). If Huang et al. (2022) elect *Ph*4CL1 as a candidate for the formation of benzoyl-CoA after feeding benzoic acid, how its RNAi showed no effect on the emission of benzenoid (Klempien et al., 2012)? Noteworthy, the expression pattern of *Ph*4CL1 had been shown to be unrelated to the rhythm of benzenoid emission (Klempien et al. 2012).

The aforementioned comments question the exclusive role of β-oxidative pathway (presented by KAT1) for the biosynthesis of benzoyl-CoA the substrate of the newly discovered benzaldehyde synthase.

Conclusion

The formation of benzoyl-CoA after feeding benzoic acid requires BZL activity, while its formation after feeding L-phenylalanine could be catalyzed either by the previously detected KAT enzyme or an un-defined BZL. The formed pool of benzoyl-CoA seems to be comparable as in both cases benzaldehyde synthase uses this pool to produce comparable amount of benzaldehyde (23.03% after feeding $^{2}H_{5}$-benzoic acid and 22.51% after feeding $^{2}H_{8}$-phenylalanine; Huang et al., 2022). The accumulation of deuterium-labeled benzaldehyde and benzylbenzoate after feeding benzoic acid shed the light on the existence of a functional BZL in petunia, which cannot be justified by the previously characterized *Ph*4CL1 or *Ph*CNL (Klempien et al., 2012).

Perspective

After feeding labeled benzoic acid, the protein that supplies sufficient benzoyl-CoA substrate to be utilized by the peroxisomal benzaldehyde synthase is to be defined. To date, petunia benzoyl-CoA is believed to be a product of a pure β-oxidative pathway in peroxisomes. On the other hand, it is still unknown how the peroxisomal pool of benzoyl-CoA is exported to the cytosol for benzylbenzoate formation by BPBT. Similar to the reported dual localization of *Hypericum* acyl activating enzyme (Singh et al., 2020), a dual targeted BZL to the cytosol and the peroxisome might be the answer for petunia.

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