Review of: "Natural Variation Meets Synthetic Biology: Promiscuous Trichome Expressed Acyltransferases from Nicotiana acuminata"

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Review of: “Natural Variation Meets Synthetic Biology: Promiscuous Trichome Expressed Acyltransferases from *Nicotiana acuminata”*

This paper is a continuation of a series of research studies executed in Robert Last’s laboratory at Michigan State University. The present study focused on acylsugar assembly in the Solanaceae family, and more specifically in a group of species that represent a range of diversity across the genus *Nicotiana*. Acylsugars (ASs) are secondary products that are characteristic of this family. AS play a defense role against pests, such as insects and fungi, they help protect plants against desiccation, and they also possess antibiotic activity. The paper explores several areas: (1) AS diversity within the genus *Nicotiana*; (2) a search for AS acyltransferases (ASATs) in*N. acuminata* that produce large numbers of diverse acyl sugars, including tiglylated acyl sugars; and (3) an *in vitro* characterization of the biochemical functions of acyltransferase enzymes produced by the newly discovered genes.

The experiments were designed meticulously and executed with great precision; thus, there is little doubt about the validity of the reported results. The authors used state-of-the-art methodology and equipment to reveal the AS diversity of species in the genus *Nicotiana*. The AS structural variants were studied using (CID) LC-MS QTOF; sugar core analysis of the intact ASs was studied by LC-MS QTOF; the phylogenetic distribution of acyl chain structural variations identified by LC-MS CID fragmentation patterns was the basis for mapping the acyl chains onto the *Nicotiana* phylogeny; acyl chain structural variations were explored by GC-MS. Trichome transcriptomic analysis in *N. acuminata* led to the identification of four acyltransferase gene candidates, of which the first and fourth were characterized by their *in vivo* and *in vitro* activities. *N. acuminata* acyltransferases 1 to 4 were shown to synthesize structurally diverse acylsugars.*In vitro* experiments were diverse and included a simple one enzyme and one substrate mix, a “one-pot” reaction with the four enzymes and multiple substrates, and cross-species ASATs mixed assays.

The major findings in this paper include the discovery of four acylsugar acyltransferases in*N. acuminata* that have a high promiscuity to synthesize a wide variety of products, including those not found in the natural trichome complement of acyl sugars. ”*In vitro* acyl sugar combinatorial analysis with *Nicotiana (acuminata*) acyltransferases showed their potential as synthetic biology tools to produce acyl sugars with varied biological activities”.

Comments:

1 Supplemental Fig. S1

1. I disagree with the labeling of acyl chain types i.e. 5, 5d, 6, 7, 8. There should be a distinction between straight and branched chains (as in Fig. S6). Label “5” could be nC5, iC5, or ai5. The last two are isomers coming from the degradation of different amino acids. Straight-chain C5 (valerate) is very rarely found in nature. In fact, we have never seen valerate as a component of plant acyl sugars. 5d, as labeled, stands for pentenoic acid, not tiglic acid. Our lab has characterized acylsugar acyl groups from numerous *Nicotiana* species, but I cannot compare with the information of Fig. S1 because the last does not contain the relevant details. Acyl sugar acyl chain composition and structures are already given for ten of species (Fig. S6). If the authors are inclined to revise Fig. S1, it would serve as a valuable reference, and should be converted to regular figure, not supplemental.
2. *N. tomentosiformis*: this species is considered to be one of the progenitor species that gave rise to cultivated tobacco, *N. tabacum*. The aiC6 is the longest (together with iC6) and major acyl group of the acylsugars that were inherited in tobacco. We have performed numerous analyses with *N. tomentosiformis*, and also found C3, nC4, and iC4, which are not given in Fig. S1. We have not seen evidence for C7 and C8 acyl groups.

Curiously, we found iC7 aiC7 groups in some of the plants grown from seeds in the pack of *N. tomentosiformis* seeds obtained from the U.S. Department of Agriculture Germplasm Resources Information Network (GRIN). Plants segregated 1:1 with

respect to the acylsugar acyl group profiles. The first profile included, in the order of

peak area abundance, the following acyl groups: aiC6> aiC5 > C2 > iC5 > C3 > nC4 > iC6. The second profile included, in the order of peak area abundance, the following acyl

groups: aiC7> iC7 > iC6 > C2 > nC4 > aiC5 > iC4 > aiC6 > C3. Neither of those two

profiles matches the chain types of *N. tomentosiformis* in Fig. S1. There is only

accession available from GRIN which excludes the possibility that the differences of the acyl profiles are due to different sources (accessions). Until further clarification, I suggest that this species be removed from Fig. S1

3. *N. tabacum* TN 90: We have found C5d (minor group), which is not reflected in Fig. S1. C6 was present as aiC6 and nC6.
4. *N. alata*: Our data are different from those in Fig. S1. We did not find evidence for C7 acyl groups in *N. alata*, but detected nC4, iC4, nC6, and aiC6 acyl groups that are absent from the figure. The difference may be caused by the source. There are four active accessions

in the GRIN database.

2. Supplemental Figure S18

There are labels **Na**ASAT**2** (S18a) and **Na**ASAT**2** (S18c and d). What enzymes are these? Is there a typo ‘Na’ instead of ‘Nac’ or does the label stand for a different species ‘Na’?

3. Discussion, line 484:

For clarity, “C5-CoA acyl chains” should be replaced with “iC5-CoA and aiC5-CoA acyl chains”.