Conversion of Unstable Compounds Can Contribute to the Auxin Pool during Sample Preparation[OPEN]

Dear Editor,

Auxin is a powerful regulator of plant growth and development (Heisler and Byrne, 2020). Accurate quantification of the main form of auxin, indole-3-acetic acid (IAA), is therefore essential for the study of auxin biology. Synthetic forms of the IAA-related compounds indole-3-pyruvic acid (IPyA) and indole-3-acetaldehyde (IAAld) have been shown to convert nonenzymatically to IAA, particularly in aqueous environments (Ernstsen et al., 1986; Tam and Normanly, 1998; Quittenden et al., 2009; Dai et al., 2013). These compounds are present in plants, and IPyA is now considered the main intermediate between tryptophan and IAA (Zhao, 2018). If substantial amounts of IPyA and/or IAAld convert to IAA during sample preparation, IAA quantification may be severely compromised. Here, a cysteamine-based derivatization method for converting IPyA and IAAld to stable forms (Novák et al., 2012) was used to demonstrate that endogenous IAA-related compounds can convert nonenzymatically to IAA during pea (*Pisum sativum*) and Arabidopsis (*Arabidopsis thaliana*) sample preparation. In derivatized samples, quantified IAA content was up to 40% lower than in underivatized controls. As a result, we recommend that harvested samples be derivatized soon after the completion of a short extraction period.

First, we compared the effects of two treatments on the breakdown of synthetic IPyA to IAA. Aliquots of synthetic IPyA were either left untreated, derivatized with cysteamine (Novák et al., 2012), or treated with the antioxidant sodium diethyldithiocarbamate (DEDTCA; Ernstsen et al., 1986; Supplemental Materials and Methods). The proportion of IAA recovered (compared with the starting weight of IPyA) was 15.7% in the control (untreated) case, 3.5% after DEDTCA treatment, and 0.5% after cysteamine derivatization (Supplemental Fig. S1). Cysteamine effectively converted IPyA to the derivatized form, known as IPyA-TAZ (Novák et al., 2012), apparently removing IPyA from the pool that can contribute to IAA. DEDTCA, in contrast to cysteamine, directly inhibited the conversion of IPyA to IAA, and in DEDTCA samples, the signal for underivatized IPyA was over twice that in control samples (Supplemental Table S1; the signal for [$^{13}C_6$] IAA acts as a standard for comparison). This indicates that <50% of the original IPyA remained intact in the control case over the ~10 h of the experiment. Another degradation product of IPyA, indole-3-carboxaldehyde (Tivendale et al., 2012), was not quantified.

Next, we tested the effects of cysteamine derivatization (Novák et al., 2012) on IAA recovery in a biological context, with and without DEDTCA. In extracts from the apical portions of pea seedlings, derivatization reduced the IAA detected with and without DEDTCA by 29% and 37%, respectively (Fig. 1). In underivatized samples, DEDTCA also significantly lowered the amount of IAA recovered. However, no difference in IAA content was detected between the two derivatized treatments (that is, with or without the antioxidant).

These results indicate that either or both of the endogenous IAA-related compounds IPyA and IAAld converted nonenzymatically to IAA in the underivatized samples and that this breakdown contributed to the free IAA content recovered. We suggest that derivatization converted these compounds to stable derivatives, preventing their conversion to IAA. Consistent with that, IPyA-TAZ was detected in all cysteamine-treated samples (Supplemental Fig. S2), but not in any of the underivatized samples. No underivatized IPyA was detected whether samples were

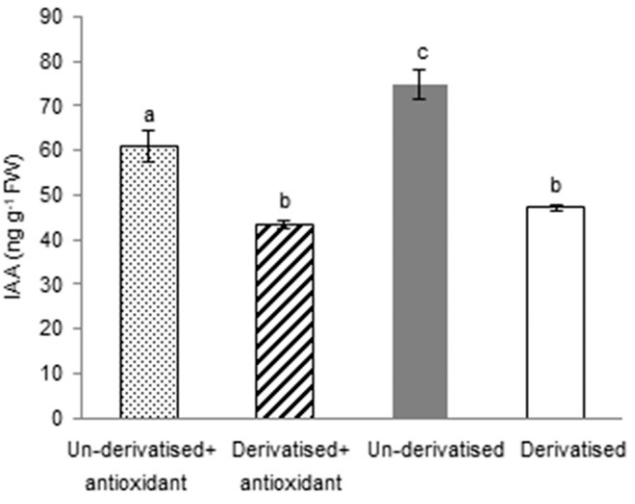

Figure 1. Effects of derivatization and of the antioxidant DEDTCA on IAA content recovered from pea apical portions. Results are means ± SE ($n = 4$) and lowercase letters denote significant differences ($P < 0.05$, Student's t test). FW, Fresh weight.

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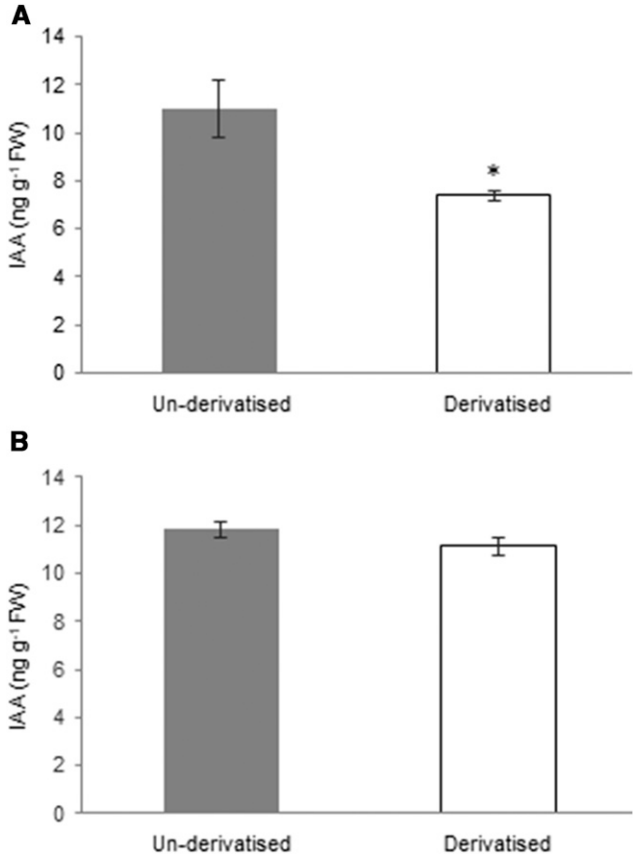

Figure 2. Effects of derivatization on IAA content recovered from Arabidopsis shoots at 10 days after germination (25 mg per sample, $n = 5$ technical replicates from a single harvest; A) and 30 days after germination (20 mg per sample, $n = 4$ technical replicates from a single harvest; B). Results are means ± SE and the asterisk denotes a significant difference at the $P < 0.02$ level (Student's t test). FW, Fresh weight.

derivatized or not. The results also demonstrate that while the antioxidant does enhance the stability of labile indoles, cysteamine-based derivatization is a more effective method than DEDTCA for reducing the contribution of unstable compounds to the IAA pool.

We next tested the model species Arabidopsis to investigate whether the results obtained in pea could be replicated in a different species. Actively developing seedlings (10 d after germination) of the Col-0 ecotype were treated in the same manner as the pea apical portions in Figure 1. Again, 33% less IAA was recovered from derivatized aliquots compared with those that were underivatized (Fig. 2A). Thus, our evidence indicates a similar propensity of cysteamine to "protect" the endogenous IAA pool from being "contaminated" by the nonenzymatic breakdown of labile IAA-related compounds in Arabidopsis and pea. However, in another experiment with older Arabidopsis plants (30 d after germination) there was no difference in the level of IAA quantified from derivatized and underivatized aliquots (Fig. 2B). Therefore, we are not suggesting that there will be a contribution to the IAA pool from labile compounds in every harvested sample.

We then tested the proposition that the postharvest breakdown of elevated IPyA in a *yuc* mutant (*yuc1yuc2yuc6*) could potentially restore the measured IAA content to wild-type levels (Won et al., 2011). YUC enzymes catalyze the conversion of IPyA to IAA in a range of plant species (Mashiguchi et al., 2011; Stepanova et al., 2011; Won et al., 2011; Zhao, 2018). We used a *yuc1* mutant allele of pea (also known as *crd-4*; McAdam et al., 2017) to monitor IAA content from derivatized and underivatized wild-type and mutant samples. Significantly less IAA was recovered from derivatized samples in both genotypes (Fig. 3). The *crd-4* mutation reduced IAA content in both derivatized and underivatized samples, although as for many *yuc* mutants, the effect was moderate (Fig. 3). The results indicate that at least for some *yuc* mutants, the relatively small effect on IAA content is not due to postharvest conversion of IPyA to IAA. Rather, we suggest that in the case of *crd-4*, downregulation of IAA conjugate formation is a more likely explanation, based on the substantial reduction of IAA-Asp content in this mutant (McAdam et al., 2017).

It is possible that cysteamine also derivatized compounds other than those that are auxin related. However, this could not have affected the detected ratio of unlabeled to labeled IAA. Indeed, even if derivatized compounds caused ion suppression of IAA signals, there could not have been differential suppression of unlabeled and [^{13}C]-labeled IAA, because [^{13}C]-labeled compounds exactly coelute with their corresponding unlabeled analyte (Berg and Strand, 2011). In any case, the peak areas of the [^{13}C]-IAA internal standard ruled out ion suppression as a factor. For example, the mean peak areas (± SE) for the internal standard from underivatized and derivatized wild-type samples in Figure 3 were 20,717 ± 1,027 and 19,831 ± 1,196, respectively (arbitrary units; $P = 0.6$).

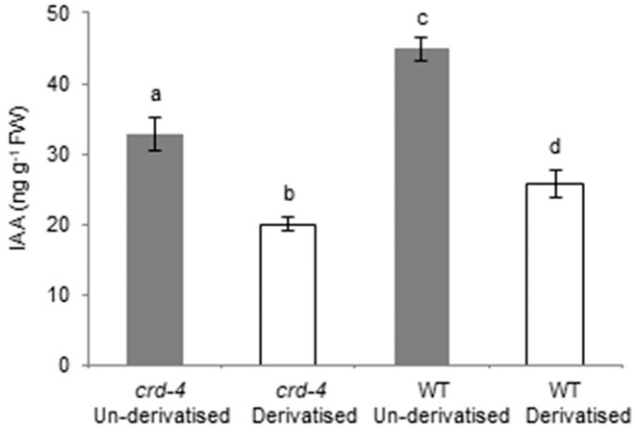

Figure 3. IAA content in *crd-4* and wild-type (WT) apical buds from derivatized and underivatized samples. Results are means ± SE of 5 biological samples and lowercase letters denote significant differences at the $P < 0.02$ level (Student's t test). FW, Fresh weight.

These results strongly indicate that unstable endogenous IAA-related compounds can convert nonenzymatically during sample preparation and contribute to the IAA pool. In pea, these compounds are IPyA and/or IAAld, as both can break down to IAA and can be derivatized by cysteamine. In Arabidopsis, another compound, indole-3-acetaldoxime (IAOx), may also contribute, since at low pH, IAOx is reported to convert to IAAld (Rajagopal and Larsen, 1972). IAOx is not found in pea (Quittenden et al., 2009). The endogenous level of these compounds, as well as the extent to which they convert to IAA rather than to alternative products, would be expected to affect the degree of contamination of the IAA pool.

Recent findings have revealed how IPyA glucosylation can modulate IAA content in response to external conditions (Chen et al., 2020), highlighting the importance of the in vivo metabolic fate of IPyA. Here, we draw attention to the in vitro fate of all auxin-related labile compounds, including IPyA. In the future, quantification of these compounds will further characterize their contributions to the free IAA pool during sample purification. It is already clear, however, that if precautions are not taken to prevent or minimize this contribution, researchers may be measuring spurious IAA in addition to genuine endogenous IAA.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Effects of derivatization and antioxidant treatment on the nonenzymatic conversion of synthetic IPyA to IAA in distilled water.

Supplemental Figure S2. Representative mass chromatogram demonstrating the presence of IPyA-TAZ in a pea extract treated with cysteamine.

Supplemental Figure S3. Procedure used for the experiment in Figure 1.

Supplemental Table S1. Peak areas of compounds detected after treatment of standard IPyA (data are in arbitrary units $\times 10^3$; see Supplemental Fig. S1).

Supplemental Table S2. MRM transitions and retention times used to monitor IAA [$^{13}C_6$]-IAA, IPyA, and IPyA-TAZ.

Supplemental Materials and Methods.

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